UNIVERSIDAD COMPLUTENSE DE MADRID
FACULTAD DE FARMACIA

TESIS DOCTORAL

Diseño, desarrollo y evaluación de formulaciones de administración vaginal para la prevención de la transmisión sexual del VIH

Design, development and evaluation of vaginal administration formulations for the prevention of HIV sexual transmission

MEMORIA PARA OPTAR AL GRADO DE DOCTOR

PRESENTADA POR

Fernando Notario Pérez

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DISEÑO, DESARROLLO Y EVALUACIÓN DE FORMULACIONES DE ADMINISTRACIÓN VAGINAL PARA LA PREVENCIÓN DE LA TRANSMISIÓN SEXUAL DEL VIH

DESIGN, DEVELOPMENT AND EVALUATION OF VAGINAL ADMINISTRATION FORMULATIONS FOR THE PREVENTION OF HIV SEXUAL TRANSMISSION

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Los resultados expuestos en la presente Tesis Doctoral han sido publicados o están pendientes de publicación en los siguientes artículos científicos:

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- NOTARIO-PÉREZ F, CAZORLA-LUNA R, MARTÍN-ILLANA A, RUIZ-CARO R, PEÑA J, VEIGA MD. Tenofovir hot-melt granulation using Gelucire® to develop sustained-release vaginal systems for weekly protection against sexual transmission of HIV. *Pharmaceutics* 2019; vol. 11, no. 3. pii: E137.
- NOTARIO-PÉREZ F, CAZORLA-LUNA R, MARTÍN-ILLANA A, GALANTE J, RUIZ-CARO R, DAS NEVES J, VEIGA MD. Design, fabrication and characterisation of drug-loaded vaginal films: State-of-the-art. *J Control Release* 2020; vol. 327, pp. 477-499.
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- NOTARIO-PÉREZ F, CAZORLA-LUNA R, MARTÍN-ILLANA A, GALANTE J, RUIZ-CARO R, SARMENTO B, DAS NEVES J, VEIGA MD. Influence of plasticisers on pH-dependent drug release and cellular interactions of hydroxypropyl methylcellulose/zein vaginal anti-HIV films containing tenofovir. [*pendiente de publicación*]
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“Si miras lejos, no ves el paso inmediato y tropiezas.
Hay que ir despacio, que no lento”

Diego Pablo Simeone

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ÍNDICE

RESUMEN

La infección por el virus de inmunodeficiencia humana (VIH) es un problema de salud pública a nivel mundial, pero su incidencia es particularmente preocupante entre las mujeres jóvenes de países de bajos y medios ingresos. Diversos factores predisponen a este grupo poblacional a estar más expuesto al contagio, tales como la violencia sexual, la pobreza y la falta de poder de decisión sobre su propia salud sexual. Son estas circunstancias las que han llevado al estudio de los microbicidas vaginales como una herramienta para prevenir la transmisión sexual del virus.

Los primeros microbicidas fueron diseñados para una administración diaria, pero la falta de frecuencia en su uso observada en ensayos clínicos llevó a la búsqueda de formas farmacéuticas que permitieran una posología más cómoda. En la actualidad, la investigación en el campo de los microbicidas vaginales sigue siendo particularmente intensa, explorándose el uso de formas farmacéuticas alternativas, la cada vez más presente aplicación de nanosistemas para la liberación de fármacos, o incluso el uso de probióticos modificados genéticamente para la expresión de sustancias con potencial viricida. A pesar de ello, el desarrollo de microbicidas vaginales aún tiene un largo camino por recorrer, como demuestra el hecho de que todavía no exista ningún producto comercializado para este fin.

En esta tesis, titulada “diseño, desarrollo y evaluación de formulaciones de administración vaginal para la prevención de la transmisión sexual del VIH”, se persigue el objetivo de desarrollar un microbicida vaginal que pueda ser autoadministrado por la usuaria y sirva como sistema de protección frente a la infección sexual por el VIH sin necesidad de cooperación por parte del hombre.

Una de las posibilidades evaluadas es el desarrollo de comprimidos vaginales mucoadhesivos de liberación sostenida de tenofovir. Tanto el quitosano como la hidroxipropilmetil celulosa (HPMC) demostraron ser polímeros adecuados para la fabricación de comprimidos con baja toxicidad y capaces de liberar el fármaco antirretroviral de manera sostenida. La combinación de estos polímeros en un mismo comprimido permite potenciar sus ventajas, pues son capaces de liberar el principio activo de una manera sostenida durante 72 horas, permaneciendo adheridos a la mucosa vaginal todo este tiempo.

La incorporación de gránulos formados por tenofovir y polímeros hidrófobos a estos comprimidos es propuesta como un recurso para prolongar la liberación del fármaco. Así, cuando el tenofovir es granulado con Eudragit® RS (proporción 1:2) y este granulado es a su vez incluido en una matriz en la que predomina el quitosano sobre la HPMC, se consigue la liberación sostenida de tenofovir durante 6 días. Por otro lado, cuando se incorporan gránulos de tenofovir y Gelucire® 43/01 es posible controlar la liberación del principio activo durante 9 días, al mismo tiempo que los comprimidos permanecen adheridos a la mucosa vaginal. En consecuencia, una administración semanal de esta formulación podría ofrecer a las mujeres una herramienta de protección frente a la transmisión sexual del VIH.

Por otra parte, los *films* son una forma farmacéutica que ofrece gran versatilidad para la administración vaginal de fármacos, adaptando la formulación a las necesidades terapéuticas. Aunque los *films* vaginales fueron inicialmente desarrollados para una liberación rápida de fármacos, con la formulación adecuada también pueden ser empleados para lograr liberación controlada. La combinación de HPMC y zeína (proporción 1:5), con la inclusión como plastificante de un 40% de polietilenglicol 400, demostró formar un *film* con excelentes propiedades mecánicas que además permitía liberar tenofovir de manera sostenida durante 5 días. Por lo tanto, este *film* podría ser una opción interesante para la prevención de la transmisión sexual del VIH.

Otra posibilidad es el desarrollo de microbicidas vaginales sensibles a estímulos, que podrían ser diseñados para aumentar la protección durante las relaciones sexuales. La idea sería desarrollar una formulación capaz de retener el principio activo en un medio ácido – como el fluido vaginal – y liberarlo cuando el medio es neutralizado – como consecuencia del pH del fluido seminal –. De este modo sería posible ofrecer protección frente a la infección en el momento necesario. Los *films* preparados con una mezcla de zeína y HPMC han sido adaptados para tal fin mediante la inclusión de diferentes agentes plastificantes. La plastificación con un 80% de una combinación de polietilenglicol 400 con ácido oleico (proporción 1:7) origina un *film* adecuado para la liberación pH-dependiente de tenofovir.

Otra opción es la incorporación de polímeros capaces de responder a los cambios de pH, como algunos de los derivados del ácido metacrílico. De este modo ha sido

demostrado que tanto Eudragit® L100 como Eudragit® S100 son excelentes polímeros para la fabricación de este tipo de *films*. Además, estas formulaciones permitían que la cantidad de fármaco asociado a las células en estudios *in vitro* fuera similar a la obtenida con el fármaco libre.

Por último, también debemos considerar el desarrollo de formulaciones de uso a demanda, que podrían ser aplicadas de manera dependiente de las relaciones sexuales (solamente necesitarían ser administrados de forma inmediatamente previa al coito para ofrecer protección). Para este propósito han sido diseñados discos vaginales basados en geles de HPMC liofilizados. Estos discos han sido desarrollados mediante la inclusión de agentes surfactantes y ciclodextrinas, para así conseguir una formulación capaz de incorporar fármacos tanto hidrófilos como lipófilos. Los sistemas que combinaban 2-hidroxipropil-β-ciclodextrina y lauril sulfato sódico demostraron ser adecuados para la rápida liberación vaginal tanto de tenofovir como de dapivirina. Por lo tanto, estos discos podrían ser una opción interesante para ofrecer protección a las mujeres frente a la transmisión sexual del VIH mediante una administración asociada a las relaciones sexuales.

Las distintas formas farmacéuticas y estrategias de prevención desarrolladas permiten que las usuarias de estos microbicidas dispongan del mayor número de alternativas posibles, para así poder adaptar la posología para una mayor comodidad de la mujer. Estas formulaciones, con el correspondiente apoyo para llevar la investigación a ensayos *in vivo*, podrían resultar en potenciales herramientas para prevenir la transmisión sexual del VIH.

SUMMARY

The infection by human immunodeficiency virus (HIV) is a global public health problem, but its incidence is particularly worrisome in women from low- and middle-income countries. Several factors predispose this population group to be more exposed to sexual acquisition of the virus, such as the frequency of sexual violence, poverty and the lack of decision-making power over their own sexual health. These circumstances have led to the study of vaginal microbicides as a tool to prevent the sexual transmission of the virus.

The first microbicides developed were designed for daily administration, but the low frequency of use led to the search for pharmaceutical forms that allow a most convenient dosage. Currently, research in the field of vaginal microbicides continues to be particularly intense, looking into alternative pharmaceutical forms, the application of nanosystems for drug delivery, or even the use of genetically modified probiotics for the release of viricidal substances. Despite this, the development of vaginal microbicides still has a long way to go, as evidenced by the fact that there are still no commercialised products for this purpose.

In this doctoral thesis, entitled “design, development and evaluation of vaginal administration formulations for the prevention of HIV sexual transmission”, the aim is to develop a vaginal microbicide that could be self-administered by women and serve as protection against sexual acquisition of HIV, without the need for male cooperation.

The development of tenofovir sustained release mucoadhesive vaginal tablets may be an effective strategy to reduce sexual transmission of HIV. Both chitosan and hydroxypropylmethyl cellulose (HPMC) proved to be suitable polymers for the manufacture of tablets with low toxicity and capable of releasing tenofovir in a sustained manner. The combination these polymers in a vaginal tablet allows enhancing the advantages that each one offers individually, since they control the release of the active ingredient for 72 hours, remaining attached to the vaginal mucosa all this time.

The incorporation of granules formed by tenofovir and hydrophobic polymers to these tablets is proposed to extend drug-release. Thus, when tenofovir is granulated with Eudragit® RS (ratio 1:2) and these granules are in turn included in a matrix in which chitosan predominates over HPMC, the tablets are capable of releasing tenofovir in a sustained manner for 6 days. Meanwhile, when granules are prepared with tenofovir and Gelucire® 43/01 it is possible to control the release of the active ingredient for 9 days, remaining the tablets attached to the vaginal mucosa. Consequently, a weekly administration of this formulation could offer to women protection against sexual acquisition of HIV.

On the other hand, films are a pharmaceutical form with great versatility for the vaginal administration of drugs, because it offers multiple options to adapt the formulation to therapeutic requirements. Although vaginal films were initially developed for immediate drug release, with the suitable design they can also be used to achieve controlled release. The combination of HPMC and zein (ratio 1:5), with the inclusion of 40% polyethylene glycol 400 as plasticiser, was shown to form a film with excellent mechanical properties that also allowed tenofovir to be released in a sustained manner for 5 days. Therefore, this film could be an interesting option for the prevention of sexual transmission of HIV.

Other choice is the development of stimulus-sensitive microbicides, which could be designed to increase protection during sexual intercourse. The aim would be to develop a formulation capable of retaining the drug in an acidic medium – such as vaginal fluid – and releasing it when the medium is neutralised – as a consequence of the seminal pH –. In this way, it would be possible to offer protection against infection just when it occurs. Films prepared with a mixture of zein and HPMC have been adapted for this purpose by including different plasticisers. The plasticisation with 80% of a combination of polyethylene glycol 400 and oleic acid (ratio 1:7) results in a suitable film for the pH-dependent release of tenofovir.

Another option is the inclusion of polymers able to modify their permeability or solubility according to the pH of the medium, such as some methacrylic acid derivatives. Thus, it has been shown that both Eudragit® L100 and Eudragit® S100 are excellent

polymers for the manufacture of these films, that achieve *in vitro* drug levels associated to cell membranes similar to free tenofovir.

Finally, it should also be considered the design of products for on demand use, which could be applied in a sexual intercourse dependent manner – would only need to be administered immediately prior to intercourse to offer protection –. Vaginal discs based on freeze-dried HPMC gels have been designed for this purpose. These discs have been developed through the inclusion of surfactants and cyclodextrins, in order to achieve a formulation capable of holding both hydrophilic and lipophilic drugs. Systems combining 2-hydroxypropyl-β-cyclodextrin and sodium dodecyl sulphate proved to be suitable for rapid vaginal release of both tenofovir and dapivirine. Therefore, these discs could be an interesting option to offer protection to women against sexual transmission of HIV through an administration associated with sexual intercourse.

The variety of dosage forms and prevention strategies developed allow users to have as many alternatives as possible, in order to adapt the dosage to greater comfort for women. These formulations, with the suitable research at *in vivo* trials, may result into potential systems to prevent the sexual transmission of HIV.

INTRODUCCIÓN GENERAL

1. Virus de inmunodeficiencia humana (VIH)

1.1. Características y serotipos

El VIH es un virus del género *Lentivirus*, de la familia *Retroviridae*. Se trata de un virus de unos 100 nm de diámetro envuelto con una membrana lipídica. Se caracteriza por presentar dos glicoproteínas en su superficie – gp120 y gp41 –, que resultan fundamentales a la hora de reconocer receptores de superficie celulares [1-3]. Precisamente la gp120 juega un papel fundamental en la infección por VIH, pues es capaz de unirse a la glicoproteína CD4 presente en la mayoría de los linfocitos T y macrófagos [4]. El virus también presenta una serie de enzimas que serán cruciales para su replicación; la transcriptasa inversa, la integrasa y la proteasa. Su genoma se compone de dos copias idénticas de RNA monocatenario. Existen dos serotipos del virus – VIH-1 y VIH-2 –, que desde un punto de vista estructural únicamente difieren en la organización de su genoma [1, 2].

1.2. Síndrome de inmunodeficiencia adquirida (SIDA)

El VIH se transmite mediante fluidos corporales, pudiendo producirse la infección a través de mucosas – incluso encontrándose éstas intactas –, a través de heridas en la piel o mediante inoculación vía parenteral [2]. Es por ello que en la actualidad las vías de infección más frecuentes son la transmisión vía sexual y entre usuarios de drogas por vía parenteral, aunque también existe transmisión materno-fetal y por transfusiones sanguíneas o trasplantes [5, 6].

El virus puede ser detectado en tejido linfático regional 1-2 días después de la infección, en ganglios linfáticos regionales tras 5-6 días y en el resto del organismo a los 10-14 días [2]. Una vez en el organismo el virus infecta a las células del sistema inmunitario, y a través de la supresión de las funciones naturales de dichas células termina produciendo un deterioro progresivo del sistema inmunitario [5]. Como es característico de todos los *lentivirus* la infección por VIH tiene un curso crónico, con un largo periodo de latencia [1]. La primoinfección por VIH puede causar algunos síntomas inespecíficos – fiebre, diarrea, cansancio, pérdida de peso, etc. – en algunos enfermos. Después, existe un periodo de latencia, cuya duración puede variar desde dos hasta más

de veinticinco años. Finalmente, cuando la inmunodeficiencia progresa, el sistema inmune se ve debilitado y aparecen enfermedades oportunistas [2].

Cabe mencionar la diferencia entre infección por VIH y SIDA [5]. El SIDA es la etapa más avanzada de la infección por VIH, en la que el sistema inmune deja de responder con efectividad y se desarrollan distintas enfermedades como consecuencia de la falta de defensas del organismo [7]. Se considera que un paciente ha desarrollado SIDA cuando se detecta una concentración en plasma menor a 200 linfocitos T CD4+/mm^3 y/o se desarrolla algunas de las enfermedades definitorias de SIDA – así clasificadas por el CDC (*centers for disease control and prevention*) – [8].

Aunque los dos serotipos del VIH terminan causando SIDA en ausencia de tratamiento antirretroviral, la infección por VIH-2 es menos virulenta – la concentración de virus circulantes en el organismo es menor – y en consecuencia precisa de más tiempo para terminar causando SIDA [1]. Los síntomas definitorios de SIDA aparecen en un tiempo medio de 10 años tras la infección por VIH-1, mientras que cuando el serotipo causante de la infección es el VIH-2 el periodo medio hasta la aparición de los síntomas es de 15 años [2].

1.3. Tratamientos antirretrovirales

Con tratamiento antirretroviral, hoy en día es posible prolongar la fase asintomática de la enfermedad aumentando la supervivencia del paciente. Con la administración continuada de estos fármacos se logra reducir la carga vírica en el organismo hasta niveles indetectables, siendo así posible mantener la inmunocompetencia y evitar el contagio [9]. Los distintos fármacos que pueden ser empleados en el tratamiento de la infección pueden ser clasificados en: inhibidores de la transcriptasa inversa análogos de nucleótidos/nucleósidos (ITIAN), inhibidores de la transcriptasa inversa no análogos de nucleótidos/nucleósidos (ITINAN), inhibidores de la proteasa (IP), inhibidores de la integrasa (II) e inhibidores de la fusión o de la entrada (IF). En la Tabla 1 se recogen los fármacos pertenecientes a cada uno de estos grupos más frecuentemente utilizados en el tratamiento del VIH [2].

Tabla 1. Clasificación de los fármacos utilizados frente al VIH.

ITIAN	Abacavir, Adefovir, Didanosina, Emtricitabina, Estavudina, Lamivudina, Tenofovir, Zalcitabina, Zidovudina.
ITINAN	Dapivirina, Delavirdina, Doravirina, Efavirenz, Etravirina, Nevirapina, Rilpivirina.
IP	Atazanavir, Darunavir, Fosamprenavir, Indinavir, Lopinavir, Nelfinavir, Ritonavir, Saquinavir, Tripanavir.
II	Dolutegravir, Elvitegravir, Raltegravir.
IF	Enfuvirtida, Maraviroc.

El tratamiento de elección en la infección por VIH-1 es la combinación de tres fármacos diferentes; dos ITIAN junto con un ITINAN, un IP o un II. Se recomienda la elección para el inicio del tratamiento de las combinaciones de emtricitabina y tenofovir alafenamida con bictegravir, dolutegravir o raltegravir, así como la combinación de abacavir y lamivudina con dolutegravir. En cuanto al VIH-2, los fármacos de los grupos ITINAN e IF se encuentran contraindicados para este serotipo, ya que el virus presenta resistencia intrínseca a estas moléculas. Respecto a los ITIAN, aunque pueden utilizarse, su barrera genética es más baja que la del VIH-1. En base a lo anterior, la terapia antirretroviral recomendada para VIH-2 consiste en la combinación de dos ITIAN con un IP o un II [10]. Sin embargo, este mismo año han surgido datos adicionales que apoyan el comienzo del tratamiento con un régimen de dos fármacos: lamividina y dolutegravir [11]. Se considera que el tratamiento es el adecuado cuando se logra la supresión viral – niveles inferiores a 50 copias/mL de plasma – de forma permanente, consiguiendo así prevenir la transmisión, evitar la aparición de resistencias y alcanzar un estado inmunológico saludable [10]. Con las combinaciones de antirretrovirales recomendadas es posible alcanzar este nivel plasmático en un plazo de 48 semanas en más del 85% de los pacientes tratados [12].

Otra decisión que resulta de gran importancia es el momento en que el paciente infectado debe iniciar el tratamiento con antirretrovirales, pues una vez iniciado debe mantenerse de forma continua e indefinida [13]. Hasta hace unos años existía cierta controversia respecto al inicio del tratamiento antirretroviral durante la infección aguda – primeros treinta días tras la infección –, pues todas las estrategias para la supresión del tratamiento indefinido fracasaron, y además no se disponía de datos suficientes para garantizar su beneficio a largo plazo. De hecho, solo se recomendaba iniciar el tratamiento cuando el paciente presentaba afectación neurológica o de cualquier otro órgano o sistema, cuando la sintomatología era prolongada, cuando existía un alto riesgo de transmisión o cuando iba acompañada de inmunodepresión celular avanzada – recuento de linfocitos T CD4+ inferior a 350 copias/µL– [10]. Sin embargo, en los últimos años se han conocido los resultados de dos ensayos clínicos – *START* y *TEMPRANO* – en los que se ha visto que tanto la prevalencia de eventos primarios como la aparición de una situación definitoria de SIDA era significativamente menor en los pacientes que iniciaban antes el tratamiento antirretroviral [14, 15]. En consecuencia, la recomendación actual es la misma tanto en la infección aguda como en la crónica; iniciar el tratamiento en el menor tiempo posible una vez confirmado el diagnóstico por VIH-1, incluso si el paciente no presenta ninguna sintomatología – excepto en aquellos pacientes cuyo organismo logre mantener niveles indetectables de carga viral plasmática de forma continua – [16, 17]. En cuanto a la infección por VIH-2, debido a que la carga viral y la progresión clínica es más lenta, la controversia sobre el momento de inicio del tratamiento es aún mayor. Además, tampoco se dispone de ensayos clínicos que permitan tomar una clara decisión al respecto [9]. Hasta que se disponga de más información la pauta a seguir es la misma que en las infecciones por VIH-1 [10].

1.4. Situación mundial y en África subsahariana

Aunque el acceso al tratamiento antirretroviral ha reducido notablemente las infecciones y la mortalidad por causa de este virus en los últimos años, la realidad es que aun hoy en día la infección por VIH sigue siendo un grave problema de salud pública a nivel mundial [6, 18]. Los últimos datos epidemiológicos publicados indican que existen 37,9 millones de personas infectadas y que cada año 1,7 millones de personas sufren el contagio del virus. El hecho de que el número total de infectados sea cada vez

mayor tiene un aspecto positivo; las mejoras logradas en el acceso a la terapia antirretroviral, que detiene la progresión de la enfermedad y alarga la vida de los pacientes infectados. Esto se refleja también en el continuo descenso en el número de muertes relacionadas con el SIDA, que fue de 770 000 en el último año [19].

Además, la situación es especialmente delicada en el África subsahariana, donde se concentran el 61% de las nuevas infecciones. En esta área existe una gran desigualdad de género, pues el 79% de las nuevas infecciones en personas de entre 10 y 19 años se producen en mujeres. Esto supone que, cada día, aproximadamente 460 chicas adolescentes son infectadas y se producen 50 muertes en este grupo poblacional como consecuencia del SIDA [20]. Aunque el uso de preservativos es un método altamente eficaz de prevenir las enfermedades de transmisión sexual, su uso se encuentra bajo el control de los hombres [21]. Especialmente en estos países, las mujeres no tienen la capacidad para negociar el uso de medidas de protección durante las relaciones sexuales, lo que hace que sean más vulnerables frente a la infección [22]. Es por ello que la situación en los países subsaharianos requeriría principalmente de un cambio en las prácticas habituales para lograr una prevención más efectiva. Las relaciones respetuosas con la mujer, el uso de preservativos, evitar el sexo intergeneracional y la poligamia, y retrasar el debut sexual de las mujeres serían medidas que ayudarían claramente a frenar la transmisión del virus [23].

Desde el punto de vista del desarrollo farmacéutico, la investigación en microbicidas vaginales ha ganado importancia en los últimos años, ya que sería una herramienta para dar poder de decisión a la mujer sobre su salud sexual. Hoy en día, entre el 47% y el 52% de las chicas y mujeres jóvenes no tienen capacidad de decidir sobre su propia salud [20]. Sin embargo, si dispusiesen de un microbicida de administración vaginal de uso cómodo y discreto que fuera eficaz para la prevención de la transmisión sexual del VIH, las mujeres dispondrían de mecanismos para estar protegidas sin necesidad de cooperación por parte del hombre [24].

2. Microbicidas frente al VIH

2.1. Definición y clasificación

Los microbicidas vaginales son formulaciones de administración tópica sobre la mucosa vaginal capaces de prevenir la transmisión de patógenos sexuales, como el VIH [21, 25]. Están diseñados para ser administrados de forma previa a las relaciones sexuales y pueden ejercer su acción inactivando al patógeno, formando una barrera entre el patógeno y las células o a través de la mejora de los mecanismos naturales de protección de que dispone el medio vaginal [26].

Según su mecanismo de acción podemos clasificar a los microbicidas en dos grandes grupos. El primero de ellos son los microbicidas sin fármacos antirretrovirales, cuyo mecanismo de acción puede ser la mejora de los mecanismos naturales de protección, la creación de una barrera que impida la entrada del patógeno en las células o la inactivación del virus antes de entrar en contacto con las células. El segundo grupo son los microbicidas cargados con fármacos antirretrovirales, que actúan inactivando enzimas virales, bloqueando así el ciclo del virus [22].

2.2. Desarrollo de microbicidas anti-VIH

2.2.1. Microbicidas sin fármacos antirretrovirales

La primera estrategia abordada fue el desarrollo de microbicidas vaginales cargados con agentes surfactantes, con la idea de que éstos fuesen capases de inactivar al virus mediante la disrupción de su membrana lipídica antes de que entre en contacto con las células del organismo [25]. El primer microbicida en ser sometido a ensayos clínicos contenía el surfactante nonoxinol-9, frecuentemente usado como anticonceptivo por sus propiedades espermicidas [27]. Sin embargo, a pesar de que tuvieran cierta actividad antiviral *in vitro*, el principal problema encontrado en los ensayos clínicos fue que estos surfactantes también son capaces de dañar la membrana de las células del organismo, promoviendo así la infección de las mismas por el virus [24, 28]. Por lo tanto, su uso como microbicida fue descartado.

Otra opción evaluada para el desarrollo de microbicidas fue el diseño de formulaciones que permitieran mejorar los mecanismos naturales de protección del

entorno vaginal. Un ejemplo es el diseño de microbicidas acidificantes, capaces de mantener el pH ácido de la vagina incluso en presencia del fluido seminal – que de forma natural alcaliniza el medio y facilita la supervivencia del virus – [25]. Esta estrategia se basa en estudios que sugieren que un medio ácido podría inhibir al VIH [29]. Varios productos fueron testados bajo esta hipótesis – tales como BufferGel® o ACIDFORM™ – pero ninguno de ellos resultó de utilidad para conseguir la protección de la mujer en ensayos clínicos [24, 30].

También se estudió el diseño de microbicidas con agentes que tuvieran la capacidad de unirse de forma específica al VIH – principalmente a la gp120 – o a las células que sufren la infección – concretamente a los correceptores quimiocina receptora de tipo 5 (CCR5) y quimiocina receptora de tipo 4 (CXCR4) –, evitando así que el virus pueda reconocer y unirse a su correspondiente receptor en las células a las que va a infectar [21]. Los polianiones y los anticuerpos monoclonales fueron ampliamente estudiados con este objetivo [31]. Sin embargo, a pesar de que muchos de estos microbicidas fueron capaces de probar su actividad *in vitro* inactivando al virus, ninguna de estas estrategias demostró efectividad *in vivo* a la hora de prevenir la transmisión sexual del VIH [32, 33]. La presencia del semen – que no es considerada en los ensayos *in vitro* – juega un papel fundamental, pues proteínas presentes en este fluido pueden disminuir la eficacia de los microbicidas [22].

2.2.2. Microbicidas con fármacos antirretrovirales

Finalmente, el desarrollo de microbicidas vaginales sufrió un cambio de tendencia. Se comenzó a evaluar como estrategia de prevención la incorporación a los microbicidas vaginales de fármacos antirretrovirales – frecuentemente utilizados para el tratamiento de la infección – [24]. Principalmente se evaluaron formulaciones cargadas con inhibidores de la entrada o con inhibidores de la transcriptasa inversa.

2.2.2.1. Inhibidores de la entrada

A priori, los inhibidores de la entrada – como el maraviroc – serían los candidatos ideales para el desarrollo de microbicidas vaginales. Si el objetivo es prevenir la infección, lo lógico sería usar fármacos que han demostrado actuar sobre los

mecanismos virales que posibilitan su entrada en las células. Concretamente, maraviroc es un antagonista de la CCR5 que se une a dicho receptor e impide que el VIH lo haga, evitando así que pueda entrar en las células [34]. Sin embargo, este fármaco no es eficaz en todos los pacientes, pues se ha comprobado que el virus también es capaz de internalizarse en las células mediante la interacción con el receptor CXCR4 [35].

2.2.2.2. Inhibidores de la transcriptasa inversa análogos de nucleótidos

Por otro lado están los fármacos que actúan a través de la inhibición de la transcriptasa inversa, que es la enzima responsable de transformar el ácido ribonucleico (ARN) viral en ácido desoxirribonucleico (ADN). Por lo tanto, estos principios activos no impiden la entrada del virus en la célula, pero sí su replicación. Dentro de esta familia de fármacos hay dos grandes grupos en función de su mecanismo de acción; los ITIAN y los ITINAN.

Los ITIAN – que fueron los primeros fármacos en demostrar actividad frente al VIH – son fármacos que bloquean la actividad de la enzima transcriptasa inversa, ya que se mimetizan con los nucleótidos y son incorporados a la cadena de ADN, causando la terminación de la misma. De esta forma previenen que el virus se replique y pueda infectar a otras células [36]. El tenofovir es sin duda el fármaco de la familia de los ITIAN más utilizado para el desarrollo de microbicidas vaginales (Figura 1). Se trata de un análogo de nucleótido que se encuentra comercializado en España como dos profármacos diferentes; tenofovir disoproxilo y teofovir alafenamida [10]. Tiene una larga semivida y lleva varios años aprobado para su uso vía oral para el tratamiento de la infección por VIH [21]. Actúa inhibiendo la transcriptasa inversa del VIH-1 desde concentraciones bastante bajas – la concentración inhibitoria en el 50% de los casos (IC_{50}) es de 1-6 µmol/L en líneas celulares linfoides y de 1,1 µmol/L en aislados de células mononucleares de sangre periférica (PBMCs) –. También tiene actividad frente al VIH-2, mostrando una IC_{50} de 4,9 µmol/L en células metalotioneinas 4 (MT-4) [37]. Su incorporación en microbicidas vaginales demostró su eficacia tanto *in vitro* como en modelos animales a la hora de inhibir la infección por el VIH [38, 39].

Los ensayos clínicos demostraron también su seguridad y ausencia de toxicidad, tanto a nivel sistémico como a nivel local sobre la mucosa vaginal, a las concentraciones frecuentemente utilizadas como microbicidas [40, 41]. Finalmente, se evaluó la eficacia de la formulación para conseguir la prevención de la transmisión sexual del virus en ensayos clínicos, y se consiguieron los primeros resultados positivos con un microbicida vaginal [25]. Concretamente fue el estudio *CAPRISA 004*, que evaluaba la eficacia de un gel de tenofovir al 1% en mujeres sudafricanas, el que consiguió demostrar que con la aplicación de dicho gel era posible reducir significativamente la infección por VIH. Para el ensayo clínico se seleccionaron 889 mujeres de entre 18 y 40 años, que no sufriesen la infección por VIH, que fueran sexualmente activas – refiriendo haber mantenido relaciones sexuales al menos dos veces en los 30 días previos al estudio – y que no estuvieran embarazadas. En un ensayo de doble ciego, la mitad de ellas recibieron el gel de tenofovir y la otra mitad un gel placebo. El gel de tenofovir demostró ser capaz de reducir la incidencia de infecciones por VIH en un 39%, aunque estos resultados estaban bastante condicionados por la frecuencia de administración. En mujeres que reportaron una alta adherencia al tratamiento profiláctico la incidencia de la infección fue un 54% menor, en comparación con aquellas que habían recibido el placebo. La baja adherencia al uso del gel – aproximadamente el 40% de las mujeres reportó una adherencia menor al 50% – se debe principalmente a la alta frecuencia de administración requerida y al hecho de no ser una pauta fija sino asociada a las relaciones sexuales. La indicación recibida por las mujeres fue la de aplicar una primera dosis del gel 12 horas antes de las relaciones sexuales y una segunda dosis 12 horas después, sin superar nunca las dos aplicaciones diarias [42].

Estudios posteriores fueron llevados a cabo con distintas formulaciones cargadas con tenofovir, pero los resultados en cuanto a su eficacia fueron bastante dispares [43]. Un ejemplo de ello es el ensayo clínico denominado *FACTS-001*, otro ensayo doble ciego, aleatorio y realizado frente a placebo con un gel de tenofovir al 1%. El ensayo se llevó a cabo con mujeres sudafricanas de entre 18 y 30 años. Sin embargo, esta vez no se encontró eficacia en la protección frente al VIH al utilizar el microbicida. Las conclusiones de este estudio indican la necesidad de desarrollar productos alternativos que no requieran una alta adherencia o que no dependan de la administración de la

usuaria [44]. El mismo hallazgo fue encontrado al realizar el ensayo denominado *MTN-003* [45]. Nuevamente, la falta de eficacia protectora del gel de tenofovir fue asociada a la baja adherencia de las usuarias a la posología recomendada, que en este caso era una aplicación diaria [46].

Otra formulación alternativa, también evaluada en ensayos clínicos, es un *film* cargado con 40 mg de tenofovir. Aunque su eficacia aún no ha sido testada, sí se ha podido comprobar que con el *film* se consiguen concentraciones de fármaco más altas en plasma y en fluido cervicovaginal, en comparación con el gel [47]. Con el fin de espaciar la posología del microbicida también fue evaluado un anillo vaginal cargado con tenofovir, consiguiendo superar satisfactoriamente los ensayos clínicos de fase I [48]. En esta ocasión, se evaluó el uso del anillo durante dos semanas. Más recientemente, este mismo grupo de investigación ha realizado un ensayo clínico evaluando el uso de los anillos vaginales durante tres meses, pero durante la fase I el microbicida no fue capaz de garantizar su seguridad. Fue observada la aparición de ulceras vaginales y una elevada actividad inflamatoria en un significativo número de mujeres tratadas con el anillo, mientras que ninguna mujer tratada con el placebo presentó esta sintomatología [49]. Aunque esta última alternativa inicialmente resolvería el problema de la baja adherencia, el problema de seguridad obliga a replantear su desarrollo.

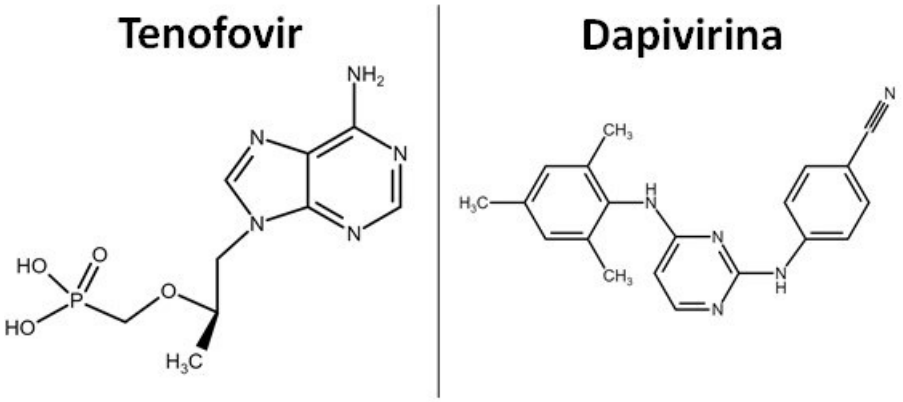

Figura 1. Estructura química de los antirretrovirales tenofovir y dapivirina.

2.2.2.3. Inhibidores de la transcriptasa inversa no análogos de nucleótidos

La otra familia de fármacos inhibidores de la transcriptasa inversa son los ITINAN, que incluye diversos fármacos capaces de unirse a un sitio alostérico en la enzima e inducir un cambio conformacional en la misma, bloqueando así su actividad [50]. A pesar de que se dispone de diversos fármacos pertenecientes a esta familia aprobados y frecuentemente utilizados para el tratamiento de la infección por VIH, llama la atención que el fármaco más habitual para el desarrollo de microbicidas vaginales es la dapivirina, un antirretroviral que no se encuentra aprobado para su uso vía oral (Figura 1). La actividad antiviral *in vitro* de la dapivirina ha sido confirmada [51] y se ha visto que su uso vía vaginal a dosis moderadas es seguro tanto en animales como en mujeres [52, 53]. Además, presenta actividad a dosis muy bajas, situándose su IC_{50} en concentraciones entre 1-5 nM. Por si fuera poco, también existen estudios que describen la capacidad de la dapivirina de acumularse dentro de las células y mantener su actividad durante varios días, así como que su potencia no se ve afectada por la presencia del moco cervical [51]. Su principal inconveniente es la pobre solubilidad que presenta en agua – inferior a 0,1 μg/mL – [54].

En cuanto a su eficacia, hay que destacar dos ensayos clínicos realizados con anillos vaginales cargados con dapivirina, por los resultados positivos que derivaron de los mismos. El primero de ellos es el estudio *ASPIRE*, que se llevó a cabo con 2629 mujeres de entre 18 y 45 años residentes en Malawi, Sudáfrica, Uganda y Zimbabue. Fueron distribuidas en dos grupos mediante doble ciego para evaluar la eficacia de un anillo vaginal cargado con dapivirina frente a otro anillo placebo [55]. La protección frente a la infección por VIH-1 con la utilización del anillo vaginal de dapivirina fue de un 37%, en comparación con las infecciones sufridas con la aplicación del placebo [56]. Por otro lado está el estudio *The Ring Study*, con características muy similares – desarrollado con 1959 mujeres de entre 18 y 45 años residentes en Sudáfrica y Uganda – y con el que se logró disminuir la incidencia de infecciones en un 31% en el grupo de estudio tratado con el anillo cargado con dapivirina [57]. Su éxito en los diversos ensayos clínicos ha convertido a este fármaco en uno de los más prometedores para el desarrollo de microbicidas vaginales destinados a la prevención de la transmisión sexual del VIH.

3. Vía vaginal

3.1. Características de la vagina

La vagina es un tubo fibromuscular de entre 7 y 10 cm de largo que conecta el cuello uterino con el vestíbulo vaginal [58]. Anatómicamente se encuentra localizada entre el recto, la uretra y la vejiga. Es la parte más externa del tracto genital femenino y desempeña funciones fundamentales en las relaciones sexuales, en el embarazo y durante la menstruación. Se trata de un órgano que en estado relajado se encuentra colapsado, entrando en contacto las paredes anterior y posterior del mismo. Sin embargo es fácilmente distensible, pudiéndose describir en consecuencia como un espacio potencial. Se ha observado también que la vagina presenta una morfología curva, con una parte inferior convexa y una parte superior prácticamente horizontal cuando la mujer se encuentra en pie [59]. Tanto el tamaño como la forma de la vagina difieren notablemente de una mujer a otra, y también existen grandes modificaciones en las características de este órgano a lo largo de la vida de la mujer [60].

La pared vaginal se compone de cuatro capas celulares distintas, que desde la más externa hasta la más interna se conocen como epitelio escamoso estratificado, lámina propia, capa muscular y capa adventicia [58]. El epitelio escamoso de cuello uterino y vagina tiene un espesor de entre 0,2 y 0,5 mm, aunque varía con la edad – se engrosa en la pubertad y se atrofia en la menopausia – [61]. Tiene una función de protección de las capas internas. Por lo tanto, es una primera línea de defensa natural frente a la infección por VIH. Esto hace que el mantenimiento de las condiciones adecuadas de esta capa celular sea esencial, ya que si la propia formulación administrada causa un daño estaría aumentando el riesgo de infección. Aunque las células epiteliales limitan la entrada de partículas con un tamaño mayor a los 30 nm – el VIH tiene un diámetro aproximado de 100 nm –, el virus es capaz de difundir mediante el mecanismo conocido como transmigración [22]. Además, el espesor del epitelio vaginal también se ve modificado a lo largo del ciclo sexual como respuesta a cambios en los niveles de estrógenos. A medida que las células van madurando se produce una migración de las mismas desde la capa basal hasta la capa superficial, que se encuentra en constante renovación [62]. Se calcula que la completa renovación celular de la capa epitelial dura

7 días. La lámina propia, que se encuentra inmediatamente bajo la capa epitelial, está formada principalmente por colágeno y elastina y se caracteriza por estar altamente vascularizada. La siguiente capa es la muscular, formada por fibras musculares lisas, y finalmente se localiza la capa adventicia, que se compone principalmente de tejido conectivo [59].

El moco cervical es otra barrera frente a infecciones, tanto de forma física como porque contiene proteínas con cierto potencial antiviral que pueden ayudar a inactivar virus, evitando así la infección [22, 63]. Se encuentra recubriendo la capa más externa de la mucosa vaginal y también tiene gran importancia en la absorción de fármacos, pues estos deben ser capaces de difundir a través de esta capa de moco antes de llegar a las células [64]. Está formado principalmente por agua y una matriz de mucina –glicoproteínas de alto peso molecular –, pero en él pueden encontrarse también enzimas, aminoácidos, proteínas, lípidos y una gran variedad de iones inorgánicos [65]. Cabe mencionar la importancia de este moco a la hora de administrar formulaciones mucoadhesivas, pues la mayoría de los polímeros mucoadhesivos son capaces de establecer enlaces con la mucina.

El fluido vaginal es el medio acuoso presente en la vagina, cuyo pH en condiciones normales se encuentra entre 3,5 y 5,5 [58]. Sin embargo, su volumen y composición puede variar notablemente según diversos factores como el momento del ciclo menstrual, la edad, el embarazo o la menopausia [66]. El pH también puede verse modificado cuando se producen infecciones. Así, la proliferación de *Gardenerella Vaginalis*, *Staphylococcus aureus* o *Escherichia coli* hacen que el pH aumente notablemente [67-69]. Tras la eyaculación también se produce un cambio rápido e importante del pH, que puede durar hasta varias horas [59]. La alcalinización del medio vaginal en presencia del fluido seminal debe ser considerada a la hora de diseñar microbicidas vaginales, pues este va a ser el pH del medio en el momento en que existe riesgo de infección.

La flora vaginal también es otro factor a considerar siempre que se administre una forma farmacéutica por vía vaginal, ya que debe ser mantenida inalterada, pues su modificación puede tener consecuencias en la salud vaginal de la mujer –

principalmente mediante el incremento de la aparición de infecciones – [62]. En el entorno vaginal se encuentra una microflora formada por bacterias tanto gram-positivas como gram-negativas, así como pequeños microorganismos anaeróbicos [63, 70]. Además, la propia flora autóctona puede jugar un papel protector frente a las infecciones, pues el ácido láctico producido por las bacterias del género *lactobacillus* ha demostrado ser capaz de inhibir la actividad de algunos agentes patógenos [22]. Son precisamente estos lactobacilos los responsables del mantenimiento del pH normal de la vagina [58].

3.2. Administración vaginal de fármacos

La administración vaginal permite una vía de acceso no invasiva que posibilita la autoadministración de las formulaciones de forma sencilla. El uso de la vía vaginal para la administración de fármacos ha sido tradicionalmente bastante empleado cuando el objetivo es un efecto local del principio activo [58]. Ha sido demostrado que existe un alto flujo sanguíneo directo desde la vagina hasta el útero – denominado efecto de primer paso uterino – que permite una alta concentración de fármacos a nivel local en útero mediante su administración vaginal [71].

Hasta comienzos del siglo XX no se llegó a considerar la vía vaginal de utilidad para lograr una absorción sistémica de fármacos [59]. Sin embargo, también es posible lograr una distribución sistémica mediante la absorción de fármacos a través de la mucosa vaginal, principalmente por a difusión simple – mientras que los fármacos hidrofílicos se absorben a través de poros presentes en la mucosa vaginal, los fármacos hidrófobos lo hacen vía intracelular – [72]. La mucosa presenta una gran superficie debido a los numerosos pliegues de su estructura, lo que es una ventaja para la absorción de fármacos [58]. Así, la superficie vaginal media suele encontrarse en el intervalo de entre 65 y 107 cm^2, lo que proporciona una gran superficie de absorción [60]. Además, el alto aporte sanguíneo en este órgano favorece también una rápida absorción de sustancias.

Las principales ventajas de la administración de fármacos vía vaginal para un efecto sistémico, en comparación con la vía oral, son que se evita el efecto de primer paso hepático y se reducen los efectos secundarios a nivel tanto gastrointestinal como

hepático [68]. En comparación con la administración parenteral, se evita el dolor causado por la perforación de tegumentos. Por último, la administración para liberación sostenida de fármacos por vía vaginal ofrece un perfil farmacocinético más estable, en comparación con la administración mediante parches transdérmicos [59].

Sin embargo, también existen algunos inconvenientes. El fluido vaginal puede suponer una barrera para la absorción de fármacos, especialmente porque sus componentes pueden ser capaces de interactuar con el principio activo [62]. Además, los cambios en su volumen y composición que tienen lugar a lo largo del ciclo menstrual pueden dar lugar a variaciones en el perfil de liberación de los fármacos [73]. Por último, los fármacos administrados mediante esta ruta deben presentar estabilidad en un pH ácido, como el encontrado en el entorno vaginal. De otra forma, podría tener lugar su inactivación antes de que puedan ejercer su acción farmacológica [67].

Entre los fármacos que son administrados por vía vaginal se encuentran antimicrobianos para el tratamiento de infecciones locales, anticonceptivos hormonales, espermicidas, principios activos para terapia de sustitución hormonal, agentes abortivos e inductores del parto [74].

3.3. Formas farmacéuticas de administración vaginal

La forma farmacéutica en que se administra un fármaco también resulta fundamental [75]. Cada forma farmacéutica es única en términos de liberación de fármaco y aceptación por las pacientes [76]. Muchos microbicidas pueden tener una baja eficacia debido a una formulación inadecuada [77]. Además, aunque está claro que los primeros factores a tener en cuenta son la seguridad y la eficacia del microbicida, también es necesario asegurar que es incorporado en una forma de administración aceptable y cómoda para las mujeres [24].

La compatibilidad fisicoquímica de los excipientes y los fármacos utilizados es esencial. Como ejemplo, aquellos fármacos que no presentan una gran estabilidad en solución acuosa no deben incorporarse en formas como soluciones o geles, sino que se debería optar por formas sólidas. Otro ejemplo son los principios activos con gran labilidad a la temperatura, cuya incorporación en anillos vaginales o en comprimidos

fabricados por moldeo o extrusión podría suponer su alteración y pérdida de estabilidad [24].

Las diferentes formas farmacéuticas de administración vaginal se pueden clasificar en líquidas, semisólidas o sólidas. Según farmacopea, los ensayos que deben realizarse sobre estas formulaciones son: uniformidad de masa o volumen extraíble – para formas líquidas y semisólidas –, uniformidad de contenido y ensayo de disolución – para formas sólidas – y uniformidad de masa – para formulaciones monodosis – [78]. Las distintas formas de dosificación que podemos encontrar para la administración vaginal de fármacos son descritas a continuación.

3.3.1. Formas farmacéuticas líquidas: Soluciones, linimentos, emulsiones y suspensiones

Se trata de formas farmacéuticas líquidas, presentadas en envases unidosis adaptados para su administración o en envases multidosis con un aplicador apropiado. Se obtienen por la disolución o suspensión del principio activo en un medio líquido. Pueden contener otros excipientes, destinados a modificar la viscosidad, la solubilidad, para ajuste de pH o agentes estabilizantes [78].

Las disoluciones son preparaciones monofásicas homogéneas en las que el fármaco se encuentra disuelto en un vehículo acuoso. Cuando el vehículo no es acuoso sino de naturaleza orgánica, se denominan linimentos. Las emulsiones son un sistema bifásico – formado por una fase acuosa y otra oleosa – de fácil reconstitución por agitación. Por último, las suspensiones son un sistema heterogéneo en el que el principio activo es fácilmente dispersable por agitación.

No son formulaciones habituales para la administración de fármacos por vía vaginal, pues presentan numerosos inconvenientes. El principal es que se produce una gran pérdida de formulación tras la aplicación, lo que hace que no sean útiles para la administración de fármacos con un estrecho margen terapéutico. Además, dicha perdida supone una incomodidad para la mujer, lo que las convierte en formulaciones poco cómodas de administrar. Por lo general se utilizan con fines de diagnóstico o irrigación, o para realizar lavados vaginales.

Por último, encontramos también las duchas vaginales. No se trata de una forma farmacéutica en sí, sino de un dispositivo para administrar las formulaciones líquidas. La farmacopea estadounidense (USP) las menciona como “irrigaciones vaginales”. Se trata de un dispositivo que permite la administración de un flujo continuo de una disolución en el interior de la vagina. Se compone de una bolsa que contiene el líquido a irrigar y un tubo que finaliza en un bulbo, destinado a la inserción de su extremo en la vagina. Aunque su uso permite administrar formulaciones líquidas con mayor comodidad, en la actualidad se encuentran claramente desaconsejadas por asociarse con un incremento de la incidencia de enfermedades de transmisión sexual, así como de endometritis, enfermedad inflamatoria pélvica o cáncer de cuello uterino [79]. Además, su uso frecuente puede conllevar modificaciones en el pH y la microflora vaginal, lo que puede desencadenar en otros problemas de salud.

3.3.2. Espumas

Según la Farmacopea europea, las espumas son sistemas dispersos heterogéneos en los que un gran volumen de gas se encuentra disperso en un líquido –que normalmente es el vehículo del principio activo –. Además, se requiere la incorporación de tensioactivos para facilitar la formación y estabilidad de la espuma [78].

En la práctica, las espumas se generan en el momento de su aplicación. Son soluciones administradas con la ayuda de un dispositivo a presión. La presencia de un gas propelente genera la espuma partiendo de la disolución del fármaco. De esta forma se consigue reducir la pérdida de formulación que ocurre al administrar formulaciones líquidas.

3.3.3. Pomadas

Las pomadas son formulaciones semisólidas formadas por una base de naturaleza oleosa en la que se disuelven o dispersan los principios activos. Pueden distinguirse pomadas hidrófilas – formadas por una base miscible en fluidos acuosos –, pomadas hidrófobas – solo pueden absorber pequeñas cantidades de agua – y pomadas que emulsionan agua – pueden absorber grandes cantidades de agua, dando lugar a una

emulsión – [78]. Se envasan en recipientes monodosis o multidosis, provistos de un aplicador adecuado.

3.3.4. Cremas

Las cremas son formulaciones semisólidas bifásicas que se componen de una fase acuosa y una fase oleosa emulsionadas. Entre los componentes de la formulación es frecuente encontrar agentes emulgentes que faciliten la formación del sistema bifásico. En función del signo de la emulsión se pueden clasificar en cremas lipófilas – fase externa oleosa – o hidrófilas – fase externa acuosa – [78]. Las características de los envases son las mismas que para las pomadas.

3.3.5. Geles

Los geles son sistemas semisólidos formados por líquidos gelificados. Generalmente están constituidos por uno o varios polímeros, que al ser disueltos o dispersos en un líquido son capaces de formar redes tridimensionales en las que largas cadenas poliméricas se conectan entre sí en puntos específicos, alcanzando un alto grado de reticulación [58]. La consistencia de la formulación va a venir determinada por la cantidad de agente gelificante. En función de la polaridad del líquido absorbido en la matriz polimérica es posible hacer distinción entre hidrogeles – aquellos que contienen un solvente polar, como el agua – y oleogeles – formados por líquidos apolares, como los aceites orgánicos – [78]. Los hidrogeles sin duda son mucho más habituales, siendo utilizados no solo como vehículos para la administración de fármacos sino también en la industria alimenticia y en ingeniería de tejidos.

La principal ventaja de los geles es que son formulaciones que permiten una cómoda y fácil administración, teniendo en consecuencia una gran aceptación por parte de las mujeres [80]. Es por ello que son probablemente la forma farmacéutica más utilizada para la administración vaginal de fármacos. Su fabricación a gran escala también es fácil y económica [75]. Por lo general, se utilizan geles cuando se quiere que el fármaco comience a ejercer su acción de forma rápida tras su administración.

El principal inconveniente de los geles para las usuarias es que con frecuencia ocurren pérdidas de la formulación, ya que parte del gel puede ser expulsado tras la administración [81]. Además de la variación en la dosis administrada como consecuencia de esto, supone una incomodidad para la mujer. También es frecuente que el gel no se distribuya de manera uniforme a lo largo de la vagina, pudiéndose no alcanzar una dosis efectiva en algunas áreas [77]. Habitualmente requieren de un aplicador para su administración que, aunque facilita dicha labor, incrementa notablemente el coste del producto [75]. Otra desventaja es que su estabilidad es menor que la de las formas sólidas, pues son más inestables frente a condiciones ambientales desfavorables [54]. Además, puesto que generalmente se utilizan hidrogeles, los fármacos con inestabilidad en solución acuosa tienen dificultades para ser incluidos en esta forma de dosificación.

3.3.6. Óvulos

Los óvulos son formas farmacéuticas sólidas monodosis. Tienen una forma generalmente ovoide y un tamaño adecuado para su cómoda administración. El principal excipiente es una base en la que se dispersan o disuelven los principios activos. Así, en función de las características de dicha base, pueden clasificarse en óvulos que se disuelven en el fluido vaginal y óvulos que funden a la temperatura corporal. Cualquiera de estos dos mecanismos va a ser el que controle la liberación del principio activo. Otros excipientes que pueden incluirse en los óvulos vaginales son diluyentes, adsorbentes, surfactantes, lubrificantes, conservantes y colorantes [78].

La fabricación se realiza por moldeo; se prepara la disolución o dispersión del principio activo – y otros componentes, si los hubiera – en el excipiente que sirve como base de la formulación y se vierte en un molde con la forma deseada. El óvulo se forma al enfriar y solidificar esta mezcla. Además de aquellos ensayos requeridos para las formas farmacéuticas sólidas vaginales, los óvulos deben cumplir el ensayo de disgregación, satisfaciendo dicho ensayo cuando se produce la completa disgregación en menos de una hora. En algunos casos, se realiza también un ensayo de resistencia a la fractura.

Por último, debemos mencionar que aunque la mayoría de los óvulos vaginales están diseñados para una liberación rápida del fármaco, también es posible preparar óvulos para liberación prolongada. Estos últimos están exentos de cumplir el ensayo de disgregación, pero deberán demostrar dicha característica en el ensayo de liberación del principio activo.

3.3.7. Comprimidos

Los comprimidos son una forma farmacéutica sólida que se obtiene generalmente por compresión de un volumen determinado de partículas. Dichas partículas son los principios activos junto con distintos excipientes – diluyentes, aglutinantes, disgregantes, lubrificantes, colorantes, etc. –. Además, se pueden preparar comprimidos con polímeros mucoadhesivos, con el objetivo de aumentar el tiempo de permanencia de la formulación en la vagina. En algunos casos se realiza un proceso de granulación de la masa pulverulenta antes de la compresión.

En función de los excipientes utilizados para su fabricación se puede modificar considerablemente el tiempo de liberación del fármaco, pudiendo conseguir desde comprimidos de rápida disolución hasta comprimidos de liberación controlada. Al igual que ocurre con los óvulos, deben cumplir el ensayo de disgregación – completa disgregación en menos de 30 minutos – salvo cuando se trate de comprimidos de liberación prolongada, en cuyo caso se requiere un ensayo para demostrar la liberación del principio activo [78].

Los comprimidos presentan numerosas ventajas. En primer lugar, son una forma farmacéutica de fabricación fácil y económica a gran escala. Además, presentan una gran estabilidad frente a condiciones ambientales adversas [75]. Su transporte y administración también resulta sencillo, y garantizan una gran precisión en la dosis administrada. La principal desventaja de los comprimidos es que, al tratarse de una forma sólida, pueden tanto causar molestias como influir en las relaciones sexuales.

3.3.8. Cápsulas

Las cápsulas vaginales habitualmente presentan similitud a las cápsulas blandas para administración oral, teniendo una forma ovoide y un aspecto liso y uniforme. En algunos casos las cápsulas pueden ser óvulos vaginales encapsulados [78]. Los ensayos requeridos por farmacopea son los mismos que para los comprimidos.

3.3.9. Tampones

Los tampones medicamentosos vaginales son formas farmacéuticas sólidas, monodosis, destinadas a su inserción temporal en la vagina. Están preparados con materiales tales como celulosa, silicona o colágeno, y se encuentran impregnados de los principios activos [78].

El principal inconveniente de esta forma farmacéutica es la frecuencia de proliferación microbiana en ellas, pudiendo dar lugar a infecciones si no se utilizan de la forma o durante el tiempo adecuados. Es por ello que tienen requisitos específicos, destinados a garantizar la calidad microbiológica de la formulación.

3.3.10. Anillos vaginales

Los anillos vaginales son dispositivos basados en una estructura polimérica, flexibles y de forma circular, que son insertados en la parte final de la vagina. Los polímeros más utilizados para la fabricación de los anillos son silicona o sus derivados, polidimetilsiloxano o etinilvinil acetato [82]. Generalmente se utilizan para la administración de fármacos lipófilos – debido a la baja difusión de los fármacos hidrófilos en estos materiales –. Los anillos vaginales son avalados principalmente por su gran potencial para conseguir la liberación sostenida de fármacos – normalmente durante semanas o incluso meses – [54].

Son autoaplicables por las usuarias y además la frecuencia de administración requerida es mucho menor que la de otras formas farmacéuticas. Los principales inconvenientes son posibles dificultades en su colocación, expulsión involuntaria, molestias o interferencia con las relaciones sexuales cuando no se encuentran correctamente colocados [54].

Aunque aún no se encuentra entre las formas farmacéuticas más frecuentes para la administración vaginal de fármacos, el rápido avance en el desarrollo de esta formulación observado en los últimos años podría hacer que, a corto plazo, su producción en masa se volviera más frecuente y económica. Además, los distintos diseños posibles de la formulación – anillos matriciales, tipo reservorio, tipo sándwich, etc. – posibilitan adaptar la formulación a la liberación deseada, e incluso simultanear la liberación de fármacos incompatibles entre sí [54].

3.3.11. Films o películas vaginales

Tradicionalmente los *films* son definidos como finas láminas poliméricas, generalmente solubles en agua, que se disuelven rápidamente una vez colocados sobre la mucosa vaginal liberando en poco tiempo el fármaco que contienen [75]. Sin embargo, también es posible encontrar *films* que no se desintegran en el medio vaginal y permiten un mayor control sobre la velocidad de liberación del principio activo [83].

Pueden ser cómodamente administrados sin necesidad de un aplicador. Además, al ser una forma farmacéutica sólida, pueden ser útiles para estabilizar fármacos que se degraden en un medio acuoso [84]. También destacan por su pequeño espesor y elevada superficie con relación a su peso, lo que hace que sean más mucoadhesivos que el resto de las formulaciones vaginales – siempre y cuando sean fabricados con los mismos polímeros, pues son éstos los que van a determinar el grado de adhesividad – [85]. La principal desventaja de los *films* es que su proceso de fabricación difiere bastante de otras formas farmacéuticas conocidas, y junto a que aún no es una forma de dosificación muy habitual, hace que su fabricación a gran escala sea más costosa y no existan muchas compañías que dispongan de la tecnología necesaria [75]. Además, su pequeño tamaño hace que sea difícil incorporar grandes cantidades de fármaco, por lo que solo resultan útiles para la administración de principios activos efectivos en pequeñas dosis.

4. Estrategias y recursos tecnológicos aplicados

4.1. Desarrollo de sistemas mucoadhesivos

La bioadhesión es la unión de dos materiales – al menos uno de ellos de origen biológico – a través de fuerzas interfaciales por largos periodos de tiempo [58]. Cuando el tejido biológico es una mucosa se suele utilizar el término mucoadhesión para denominar a este proceso.

Las formulaciones mucoadhesivas permiten alargar el tiempo de retención de la forma farmacéutica en el lugar de acción, permitiendo así la posibilidad de obtener la liberación sostenida del principio activo, que en consecuencia requeriría de una menor frecuencia de administración [58, 86]. Además, puesto que el fármaco será liberado en el espacio más cercano a la mucosa, la absorción y biodisponibilidad del principio activo también se verán mejoradas [87].

4.1.1. Teoría de la mucoadhesión

Diversas teorías han intentado dar una explicación al proceso de mucoadhesión por medio de mecanismos electrónicos, mecánicos, de fractura, de adsorción, de humectación y de difusión [58]. Lo más lógico es pensar que el proceso de adhesión a la mucosa vaginal vendrá determinado por el conjunto de estos procesos, pudiendo influir todos ellos en las propiedades adhesivas de la formulación [88].

En cualquier caso, el proceso consta de una primera etapa – de contacto –, en la que se crea un contacto íntimo entre la mucosa y la formulación, y una segunda etapa – de consolidación –, en la que tiene lugar la interpenetración de componentes de un sistema en el otro [89]. Si la formulación se encuentra en estado sólido es necesario que previamente se produzca la humectación de la misma para que ocurra este contacto íntimo. Cuando las cadenas de los polímeros que constituyen la forma farmacéutica penetran en la mucosa que recubre la vagina se produce la adhesión. Del mismo modo, las cadenas de la mucina presente sobre la mucosa vaginal también se interpenetran en la formulación. Cadenas de bajo peso molecular y la solubilidad del polímero en el moco vaginal van a facilitar esta mucoadhesión [58]. Por último, se forman enlaces entre las

cadenas de polímero y las cadenas de la mucina, tales como fuerzas de Van der Waals o enlaces de hidrógeno [90], que estabilizan la interacción.

4.1.2. Características de los polímeros bioadhesivos

Los sistemas bioadhesivos se basan en la inclusión en su composición de polímeros que posean la capacidad de establecer interacciones con el tejido biológico. Polímeros de naturaleza tanto sintética como natural han sido frecuentemente utilizados con fines bioadhesivos. Hay que considerar no solo las propiedades adhesivas del polímero, sino también su capacidad de hinchamiento en el fluido vaginal y cómo van a verse afectados por el pH ácido del medio vaginal.

Numerosas características del polímero van a favorecer la mucoadhesión [91]. La presencia de grupos hidroxilos o ácidos que puedan formar enlaces de hidrógeno es posiblemente el mecanismo que va a determinar en mayor medida la adhesividad de un polímero a la mucosa [92]. Evidentemente es importante también que dichos grupos funcionales se encuentren expuestos al exterior, pues si existe impedimento estérico se va a dificultar la formación de los enlaces. Por otro lado, las cargas iónicas superficiales pueden formar enlaces electrostáticos con las cargas negativas que presenta la mucina a pH neutro. Además, la presencia de estas cargas en el polímero puede jugar un papel en la hidratación del sistema polimérico [93]. El peso molecular del polímero también tiene una influencia importante sobre la capacidad mucoadhesiva [89]. Un bajo peso molecular favorece la interpenetración de cadenas en la mucina, pero como consecuencia el gel formado va a disolverse fácilmente. Por otro lado, un peso molecular elevado va a dificultar la hidratación del polímero, disminuyendo su interacción con la mucosa. Es por ello que un peso molecular intermedio parece lo más adecuado para diseñar formulaciones mucoadhesivas [94]. La flexibilidad de las cadenas del polímero es otra característica que va a favorecer la interpenetración en la mucosa, aumentando la fuerza de la unión [95]. Por último, también se favorece la mucoadhesión con una baja tensión superficial, que facilita la extensión sobre la mucosa.

4.2. Sistemas de liberación sostenida de fármacos

El desarrollo de microbicidas de liberación sostenida de fármacos antirretrovirales es claramente la estrategia más estudiada para solventar los problemas de adherencia al tratamiento profiláctico observados en los ensayos clínicos. La idea es clara; si el fármaco es liberado durante mayores periodos de tiempo, serán requeridas menor número de aplicaciones y en consecuencia será más cómodo para la paciente cumplir con la posología, lo que se traduciría en una mejor adherencia [96]. Además, las formulaciones de liberación controlada logran disminuir los efectos adversos, manteniendo una concentración efectiva y sostenida del fármaco que evita el pico de concentración obtenido con las formulaciones de liberación convencional [97].

En lo que se refiere a la vía vaginal, la liberación sostenida de fármacos se ha conseguido mediante múltiples mecanismos de liberación – controlando la difusión del principio activo, mediante la erosión progresiva de la formulación, etc. – que principalmente han sido aplicados a formulaciones sólidas [98]. El mecanismo más habitual para conseguir liberación prolongada de fármacos en la vía vaginal es el de utilizar matrices inertes hidrófobas que permanezcan inalteradas mientras el fármaco difunde al medio. El ejemplo más claro son los anillos vaginales tipo reservorio, como pueden ser la mayoría de los anillos desarrollados para la liberación sostenida de dapivirina en la prevención de la transmisión sexual del VIH [99-101].

El control de la liberación mediante la difusión sostenida del fármaco es también el mecanismo utilizado en otro tipo de sistemas; las formulaciones recubiertas. En este caso, en lugar de ser la matriz la que controla la difusión del principio activo al exterior es una membrana externa la responsable de modificar la salida del fármaco. Aunque no ha sido un recurso muy utilizado para la vía vaginal, encontramos el ejemplo de comprimidos vaginales de vitamina C recubiertos de silicona, destinados al tratamiento de la vaginosis bacteriana [102]. Este sería también el mecanismo de control de la liberación de principios activos incluidos en anillos vaginales tipo sándwich. En estos sistemas el fármaco se encuentra disperso en una matriz que apenas controla la difusión, pero el núcleo del anillo se encuentra recubierto por una membrana que limita

la difusión del principio activo [103]. El ejemplo más claro son los anillos vaginales anticonceptivos ya comercializados que liberan etonogestrel y etinilestradiol.

También es posible optar por encapsular directamente el fármaco en un polímero que dificulte su difusión – por ejemplo, formando nanopartículas – y posteriormente incluirlo en una matriz que simplemente va a actuar como vehículo. Este es el caso de nanopartículas de alginato cargadas con el antifúngico anfotericina B, que posteriormente fueron incluidas en un gel que sirve como vehículo para su administración [104]. Pero el tamaño de las partículas que van a modular la liberación puede ser mayor. Encontramos un ejemplo de supositorios para la liberación sostenida de progesterona en los que el principio activo es granulado junto con sustancias hidrófobas – principalmente derivados del ácido metacrílico – y estos gránulos son posteriormente dispersados en el supositorio [105].

Otra alternativa para la liberación sostenida es la de utilizar sistemas matriciales hidrófilos. En estos casos, es la erosión o la disolución del polímero que constituye la matriz el principal mecanismo de liberación del fármaco. Una alternativa cada vez más empleada para la vía vaginal son los sistemas capaces de sufrir una gelificación *in situ*. En este caso, la velocidad de liberación del fármaco depende de varios de los mecanismos anteriormente comentados. En primer lugar, es necesaria la imbibición del medio acuoso en la matriz polimérica, que va a estar limitada por la velocidad de difusión del líquido y por la geometría de la matriz. A continuación, el líquido va a causar la gelificación del polímero, modificando en consecuencia las dimensiones del sistema. El fármaco, que es capaz de disolverse en el medio acuoso embebido en la formulación, comienza a difundir al exterior a través del gel formado. Por último, la disolución o erosión de dicho gel en el medio vaginal también va a condicionar una liberación más rápida del principio activo [98]. Este es el caso de los comprimidos vaginales desarrollados para una liberación sostenida de *Curcuma comosa*, que gracias a la presencia de hidroxipropilmetil celulosa (HPMC) son capaces de gelificar en presencia del fluido vaginal y liberar el principio activo de manera sostenida [106]. Del mismo modo, es posible encontrar ejemplos de comprimidos en los que este mismo mecanismo se consigue con otros agentes gelificantes [107-109].

Por último, también se han desarrollado otras formulaciones más sofisticadas, como los sistemas osmóticos. Encontramos el ejemplo de unos comprimidos para la liberación sostenida del antirretroviral IQP-058. El comprimido está constituido por una matriz de hidroxipropil celulosa, en la que se dispersa el fármaco, y una cubierta semipermeable con un orificio. La membrana que recubre al comprimido permite la entrada de agua, que va a causar la gelificación del derivado celulósico. Este gel – en el que se encuentra el fármaco – no es capaz de difundir a través de la membrana, pero sí encuentra salida al exterior a través del orificio presente en la misma. Es por ello que, a medida que el agua va penetrando, el gel va aumentando su volumen y se ve forzado a salir al exterior, liberando así el fármaco de manera progresiva [110].

4.3. Sistemas de liberación pH-dependiente de fármacos

Los microbicidas vaginales para la prevención de la transmisión sexual del VIH han sido tradicionalmente diseñados para un uso continuado, liberando el fármaco desde el momento de su administración. Sin embargo, puesto que únicamente se requiere su actuación cuando tienen lugar las relaciones sexuales – que es cuando la mujer va a estar expuesta a la infección –, existe también la posibilidad de diseñar microbicidas inteligentes o también denominados sensibles a estímulos [111]. Estos microbicidas serían administrados en cualquier momento previo a las relaciones sexuales, pero tendrían la capacidad de retener el fármaco, evitando su liberación al medio vaginal. Durante las relaciones sexuales, y más concretamente en el momento en que tuviese lugar la eyaculación, la formulación sería capaz de modificar su estructura – debido a factores como el pH o las enzimas presentes en el líquido seminal – y liberar el fármaco, consiguiendo así la inhibición de la infección viral [112].

Las formulaciones de liberación pH-dependiente son el sistema sensible a estímulos más estudiado para la administración vaginal. El pH vaginal en condiciones normales se encuentra en un intervalo de entre 3,5 y 5,5, y en presencia del fluido seminal se alcaliniza – pudiendo aumentar por encima de 7 – [113]. Para la fabricación de este tipo de microbicidas es necesario utilizar polímeros sensibles a pH, capaces de disolverse o de degradarse únicamente en un pH alcalino [114]. Estos sistemas son formulados con polímeros que contienen grupos ionizables que los hacen capaces de aceptar o donar

iones en función del pH del medio, modificando así la solubilidad o la conformación del sistema [112]. El propio cambio conformacional del sistema puede provocar la liberación del fármaco incluido cuando éste se encuentra físicamente atrapado. Otra posibilidad es que el fármaco se encuentre químicamente enlazado al polímero, y es solo cuando dicho enlace es degradado cuando se produce la liberación [115].

4.4. Sistemas de liberación acelerada de fármacos

Otra alternativa es la de diseñar microbicidas con los que se acelere la liberación del fármaco y su disolución en el fluido vaginal, para que pueda comenzar a ejercer su acción lo más rápidamente posible – lo ideal serían unos pocos minutos –. La aplicación de estos microbicidas sería a demanda y de forma coito-dependiente, justo antes de mantener relaciones sexuales, para conseguir una alta concentración del fármaco de forma rápida y así disponer de protección inmediata frente a la infección [116]. Un ejemplo de esto son unos *films* vaginales cargados con IQP-0528 que fueron desarrollados para conseguir la liberación completa del principio activo en media hora, consiguiendo además liberar más del 50% del fármaco en tan solo 10 minutos [117]. En algunos casos se incluyen agentes para favorecer la disgregación de la formulación [109, 118], mientras que en otros es necesario incluir agentes que aumenten la solubilidad – principalmente debido a la poca solubilidad en el medio vaginal del fármaco empleado – [119].

5. Principales materias primas empleadas

5.1. Ingredientes formadores de matrices

Los polímeros utilizados en la fabricación de formas farmacéuticas de administración vaginal deben garantizar la ausencia de irritabilidad y toxicidad, tanto a nivel local como sistémico [83]. Pero también hay que tener en cuenta que el polímero o los polímeros utilizados van a ser el principal modulador de la liberación del fármaco, por lo que es necesario elegir aquellos que permitan obtener la velocidad de liberación deseada. Si se busca que la formulación sea mucoadhesiva también es necesario utilizar polímeros que tengan la capacidad de establecer enlaces con la mucosa, para así aumentar el tiempo de retención de la formulación. El origen de los polímeros puede ser muy diverso, encontrando tanto polímeros naturales como sintéticos o semisintéticos. Los polímeros utilizados para la fabricación de las formulaciones desarrolladas en la presente tesis son detallados a continuación.

5.1.1. Hidroxipropilmetil celulosa (HPMC)

La celulosa es la biomolécula más abundante del planeta, pues constituye el principal componente estructural de las plantas. Sus derivados son obtenidos mediante modificaciones químicas de la celulosa, y así es posible conseguir compuestos con diferentes propiedades [120].

Entre los éteres de celulosa con aplicaciones farmacéuticas destaca la HPMC, un polímero semisintético en el que los átomos de hidrógeno de los grupos hidroxilos son sustituidos con grupos metilos e hidroxipropilos (Figura 2). Sus propiedades fisicoquímicas se ven modificadas en esta reacción, pues la temperatura de transición vítrea, la solubilidad o la viscosidad en disolución aumentan a medida que se incrementa el número de sustituciones. Tiene carácter no iónico, es biodegradable y soluble en agua [121]. Se comercializan HPMC con distintos pesos moleculares (en el intervalo de entre 10 000 y 1 500 000 Da), por lo que es importante elegir la más adecuada en función de la aplicación que se le quiere dar. Un mayor peso molecular va a implicar mayor viscosidad en disolución, lo que generalmente se traduce en un mayor control sobre la liberación del fármaco. Se trata de un excipiente frecuentemente utilizado en la

formación de matrices destinadas a la liberación sostenida y como agente de recubrimiento en formas farmacéuticas de administración oral, tópica y oftálmica. Disoluciones de HPMC, en concentraciones del 2% al 5% pueden ser utilizadas como aglutinante para procesos de granulación. En formulaciones líquidas, se aprovecha su utilidad como espesante, emulsificante, estabilizante o como agente suspensor. Este polímero destaca también por sus propiedades mucoadhesivas – gracias a la capacidad de establecer enlaces de hidrógeno con la mucosa –, que le permiten permanecer adherido durante varios días sin presentar efectos irritantes [122].

Su uso en formas farmacéuticas de administración vaginal hasta la fecha ha sido prolífero, pudiendo encontrarse principalmente en geles mucoadhesivos, tanto en fresco [123, 124] como liofilizados [125], pero también en formulaciones sólidas como comprimidos [26, 126] o *films* vaginales [127, 128].

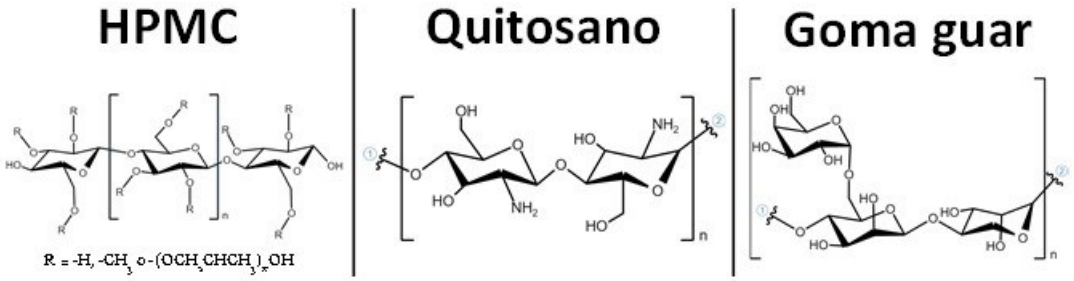

Figura 2. Estructura química de los polímeros hidroxipropilmetil celulosa (HPMC), quitosano y goma guar.

5.1.2. Quitosano

El quitosano es un polímero de origen natural que se obtiene a través de la desacetilación parcial de la quitina, un polisacárido que se encuentra formando parte del exoesqueleto de los crustáceos [129]. Es un copolímero de N-acetilglucosamina y glucosamina (Figura 2) [130]. Para su obtención la quitina es tratada con álcalis fuertes, provocando el reemplazo de los grupos acetamida por grupos amino. Esta modificación hace que el polímero tenga mayor solubilidad y reactividad [131].

Tiene carácter catiónico y se caracteriza por ser biocompatible, biodegradable y soluble en medios acuosos con pH ácido [132]. Además, posee propiedades

mucoadhesivas gracias a que presenta grupos hidroxilo, capaces de unirse a la mucosa mediante enlaces de hidrógeno, y grupos amino, que son protonados en medio ácido y pueden unirse a superficies cargadas negativamente, como las mucosas [127]. También posee propiedades antimicrobianas que podrían actuar como coadyuvantes en la prevención de enfermedades de transmisión sexual [133, 134].

El peso molecular del quitosano influye sobre la velocidad de liberación de fármacos cuando este polímero es utilizado en sistemas matriciales. Así, cuanto mayor es el peso molecular más lenta será la liberación del principio activo. Este comportamiento viene determinado por la menor solubilidad del quitosano cuando el peso molecular es elevado, pero también se debe a que cuando gelifica lo hace formando un gel de mayor viscosidad [135]. También se ha referido una relación entre el mayor peso molecular del quitosano y una mayor adhesión a la mucosa [136]. Sin embargo, esta relación no está perfectamente definida, pues otros investigadores han observado un descenso en la interacción mucina-quitosano a medida que aumenta el peso molecular [137].

También debemos mencionar que algunos investigadores han descrito el potencial del quitosano para mejorar la absorción de sustancias a través de la mucosa. Este efecto se debería a una interacción de las cargas positivas del polímero con las células de la membrana epitelial de la mucosa, que tiene como consecuencia la reorganización de la disposición estructural de dichas células [138]. De esta forma tendría lugar la apertura de uniones estrechas entre las células que favorecería la absorción paracelular a través de la membrana [136].

Encontramos una gran variedad de formulaciones vaginales basadas en quitosano; desde aquellas más habituales como geles [139, 140], comprimidos [26, 141] o *films* [142, 143], hasta otras menos frecuentes como las nanofibras [144] o los tampones [145].

5.1.3. Goma Guar

La goma guar es un polímero natural, más concretamente un heteropolisacárido extraído del endospermo de las semillas de *Cyamopsis tetragonoloba* – una planta de la familia de las leguminosas – [146]. Consiste principalmente en una cadena lineal formada por unidades de β-D-manopiranosilo, con cadenas laterales formadas por α-D-galactopiranosilo [147] (Figura 2).

Es soluble en agua, insoluble en etanol, biocompatible y biodegradable. Se caracteriza por ser uno de los polímeros naturales hidrosolubles de mayor peso molecular. Esto va a tener una gran influencia sobre su capacidad de gelificar, pues puede absorber una gran cantidad de agua – hasta quince veces su peso –, dando lugar a un gel muy viscoso incluso a bajas concentraciones [147]. La viscosidad de dicho gel va a depender de la longitud de las cadenas de galactomanano. Es por ello que en el medio vaginal – donde el pH ácido causa la hidrólisis de estas cadenas – se reduce la viscosidad de dicho gel. Este hecho puede resultar positivo para su administración vía vaginal, pues favorece su extensibilidad. Además, dado su uso habitual como protector de la mucosa gástrica, es posible que su administración vía vaginal también ocasione un efecto protector sobre la mucosa vaginal. También puede ser considerado un polímero mucoadhesivo, debido a su capacidad de formar enlaces de hidrógeno con la mucosa. De hecho, un estudio ha demostrado que su adhesividad es similar a la mostrada por la HPMC [148].

Los geles de goma guar tienen la característica de tener velocidades de erosión y disolución bastante lentas, lo que hace que mantenga su integridad por periodos de tiempo prolongados [149]. En consecuencia, han sido estudiados para la liberación prolongada de fármacos. Respecto a su uso para la vía vaginal, podemos encontrarlo en geles bioadhesivos [148, 149] o comprimidos vaginales [150, 151].

5.1.4. Polimetacrilatos: Eudragit®

La marca Eudragit® comprende un amplio grupo de polimetacrilatos – polímeros derivados del ácido metacrílico –. El método de obtención es mediante polimerización de unidades de ácido acrílico, ácido metacrílico y sus ésteres [152]. Además, pueden ser sintetizados con características catiónicas, aniónicas o no iónicas [153]. Son polímeros sintéticos diseñados principalmente para la obtención de cubiertas funcionales, ya que algunos de ellos son capaces de modificar sus características en función del pH del medio al que se ven expuestos [154].

La permeabilidad y solubilidad de estos derivados acrílicos va a verse modificada en función de los grupos funcionales que lo constituyen, pero generalmente son solubles en solventes orgánicos o soluciones alcohólicas. Además son completamente biocompatibles, pues no presentan toxicidad ni son irritantes para las mucosas. También son conocidos sus propiedades bioadhesivas, que se podrían deber tanto a la formación de puentes de hidrógeno como a los enlaces electrostáticos formados por los grupos amonio cuaternario cuando se encuentran protonados [155-157].

El uso hasta la fecha de estos polímeros en formulaciones de administración vaginal se limita principalmente a la elaboración de nanopartículas [158, 159], nanocápsulas [160], micropartículas [161] o microesponjas [162] de Eudragit® cargadas con el fármaco a administrar, que posteriormente son incorporadas en el vehículo adecuado para su aplicación.

Los Eudragit® utilizados en la presente tesis y su composición son: Eudragit® RL – copolímero de amonio metacrilato y metil metacrilato –, Eudragit® RS – copolímero de amonio metacrilato y etil metacrilato –, Eudragit® L – copolímero de ácido metacrílico y metil metacrilato en proporción 1:1 – y Eudragit® S – copolímero de ácido metacrílico y metil metacrilato en proporción 1:2 – (Figura 3) [132].

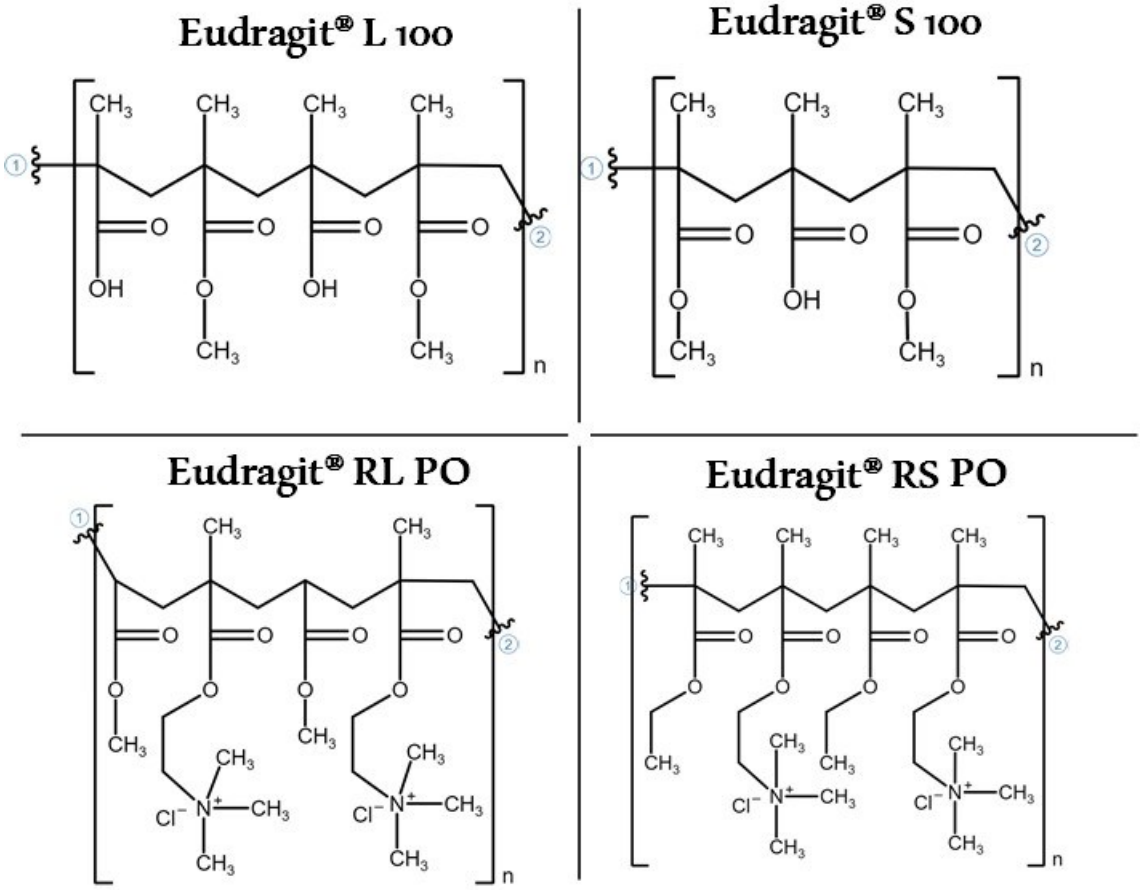

Figura 3. Estructura química de los compuestos comercializados como Eudragit® L100, Eudragit® S100, Eudragit® RL PO y Eudragit® RS PO.

5.1.5. Zeína

La zeína es una proteína obtenida del maíz que está formada por diversos aminoácidos, principalmente hidrófobos o neutros – alanina, prolina, leucina, etc. –, pero también algunos con restos polares, lo que le confiere un carácter anfifílico. Sin embargo, se ha observado que en su superficie prevalecen claramente los dominios hidrófobos sobre los hidrofílicos [163]. La estructura completa de la zeína puede a su vez dividirse en cuatro fracciones: α-zeína – que constituye la mayoría de la estructura de la proteína, aproximadamente el 80% –, β-zeína, γ-zeína y δ-zeína. [164] La fracción más hidrofóbica de las cuatro es la γ-zeína, seguida de δ-zeína, α-zeína y β-zeína, en ese orden [165]. Aunque su disposición estructural no ha sido clarificada existen varias hipótesis sobre la estructura tridimensional de la proteína. La más apoyada

posiblemente es que adquiere una conformación denominada α-hélice, en la que nueve unidades homólogas se unen de forma antiparalela mediante enlaces de hidrógeno (Figura 4) [166].

Es estable frente a las modificaciones en el pH, prácticamente insoluble en agua y soluble en soluciones alcohólicas diluidas [167]. Es biocompatible y biodegradable, garantizando la ausencia de toxicidad de las formulaciones en las que es incorporada [130]. También posee una alta estabilidad térmica, siendo posible aumentar la temperatura hasta los 280 °C sin que eso altere la estructura [168]. Aunque sus propiedades adhesivas no han sido estudiadas en profundidad, es previsible que pueda unirse a la mucosa tanto mediante enlaces de hidrógeno como a través de fuerzas electrostáticas, debido a la gran cantidad de grupos funcionales presentes en la secuencia aminoacídica que conforma la proteína.

Su carácter anfifílico le otorga excelentes propiedades de autoensamblaje, lo que ha motivado el estudio de su aplicación para la obtención de *films* [164]. Además, puede captar una pequeña cantidad de agua e hincharse ligeramente, lo que hace que también sea posible su uso en la obtención de matrices para liberación controlada [169]. Sin embargo, su potencial para la fabricación de formas de dosificación vaginales apenas ha sido explorado. Hasta la fecha, apenas podemos encontrar nanopartículas preparadas con zeína destinadas a su administración vaginal [170].

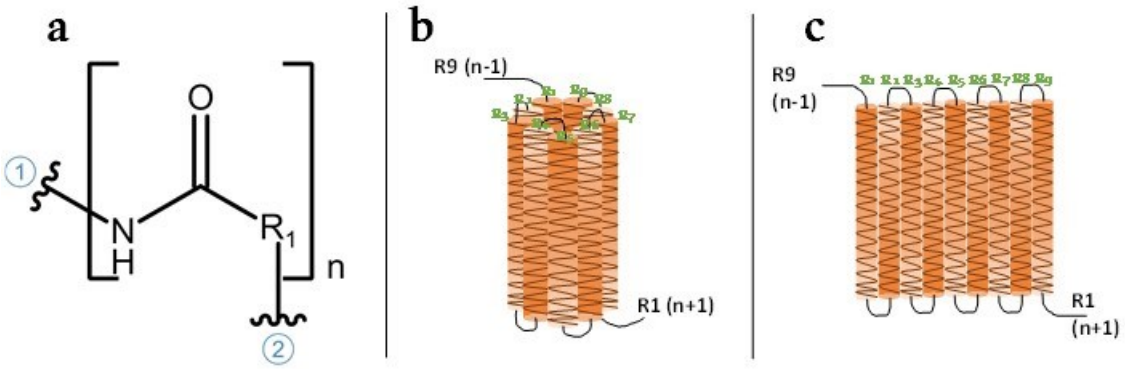

Figura 4. Estructura química de la proteína zeína (**a**) y algunas disposiciones tridimensionales propuestas de las nueve unidades homólogas; modelo cilíndrico (**b**) y modelo en cinta (**c**).

5.1.6. Gelucire®

Gelucire® es el nombre bajo el que se comercializan una serie de excipientes anfifílicos con diferentes aplicaciones en función de sus características [171]. Se encuentran disponibles numerosos tipos de Gelucire® con distinta temperatura de fusión y balance hidrófilo-lipofílico (HLB), lo que posibilita elegir entre una amplia gama de opciones en función de las características deseadas [172]. La mayoría de los Gelucire® con bajo HLB se emplean en la fabricación de matrices para liberación sostenida de fármacos. En la presente tesis son utilizados dos tipos de Gelucire®: 43/01 y 39/01, compuestos por mezclas de ésteres de mono-, di- y triglicéridos de ácidos grasos.

Gelucire® 43/01 tiene un valor HLB de 1, lo que le otorga un marcado carácter lipófilo, y una temperatura de fusión de 43 °C. Habitualmente se emplea para la protección de principios activos sensibles a la luz, la oxidación o la humedad [173]. Ha sido utilizado principalmente fabricando gránulos para numerosas aplicaciones, tales como la obtención de sistemas flotantes o de liberación prolongada [174, 175]. Sin embargo, también es posible la obtención de matrices formuladas con este excipiente mediante su fusión y posterior solidificación [176, 177]. Por otro lado, Gelucire® 39/01 es igualmente lipófilo, pero presenta una temperatura de fusión ligeramente inferior – 39 °C – y más próxima a la temperatura corporal. Los usos son muy similares a los mencionados para el Gelucire® 43/01, pudiendo encontrar sistemas flotantes y de liberación modificada [173, 178], pero además ha sido empleado para la obtención de sistemas bucodispersables [179].

Los dos tipos de Gelucire® utilizados en esta tesis no han sido previamente utilizados para la administración vaginal de fármacos. Las únicas referencias que encontramos en la literatura relativas al uso de Gelucire® como excipiente en formulaciones vaginales incluyen matrices obtenidas con Gelucire® 50/13, solo o en combinación con Gelucire® 44/14 [180, 181].

5.2. Plastificantes utilizados en los *films* vaginales desarrollados

La preparación de *films* vaginales requiere frecuentemente del empleo de agentes plastificantes que mejoren las propiedades mecánicas de los polímeros. Normalmente se utilizan moléculas orgánicas de pequeño tamaño que tengan la capacidad de colocarse entre la red polimérica y disminuir la tensión entre las cadenas [182]. Por supuesto, la ausencia de toxicidad del plastificante y su compatibilidad con los polímeros debe ser considerada en el momento de su selección [164]. Y es que, aunque se conozcan una gran cantidad de sustancias con propiedades plastificantes, solo unas pocas de ellas han sido aprobadas para aplicaciones farmacéuticas.

5.2.1. Polialcoholes: glicerol y polietilenglicol

Los polioles o polialcoholes son sin duda los plastificantes utilizados con más frecuencia en la fabricación de *films* [183]. Su estructura puede ser más o menos compleja, pero se trata siempre de un alcohol con un número variable de grupos hidroxilos. Dado su carácter polar presentan una excelente compatibilidad con los polímeros hidrófilos, que son los más frecuentemente empleados para la vía vaginal. Sin embargo, también son compatibles con sustancias de naturaleza anfifílica.

El glicerol es el polialcohol con la estructura más simple, que se caracteriza por ser altamente hidrofílico y tener un bajo peso molecular. Algunos autores han observado que la eficiencia de plastificación es mayor cuanto menor es el peso molecular del poliol utilizado – ya que así las moléculas de plastificante tienen mejor acceso entre las cadenas poliméricas –, y es por eso que el glicerol es posiblemente el plastificante más frecuentemente empleado en la fabricación de *films* [184]. En consecuencia, es posible encontrar numerosas referencias de su utilización para la plastificación de *films* de administración vaginal [159, 185, 186]. Además, es un plastificante económico, biocompatible, biodegradable y con una gran estabilidad térmica [163].

Los polietilenglicoles son otros polialcoholes, de mayor tamaño y con más grupos hidroxilos libres que el glicerol. Se trata de un polímero sintético, de cadena linear y obtenido como derivado del petróleo. Tiene un carácter fuertemente hidrófilo y es

compatible con una gran cantidad de polímeros [188]. Además, se comercializan diferentes tipos de polietilenglicol con una gran variedad de pesos moleculares, lo que posibilita elegir el más adecuado para las necesidades de cada formulación [187]. Al igual que ocurre con el glicerol, numerosos *films* vaginales han sido diseñados con este plastificante [189-191].

5.2.2. Ácidos orgánicos: ácido láctico

Los ácidos orgánicos – ácido láctico, ácido cítrico, ácido tartárico, etc. – son otro grupo de sustancias hidrófilas que han sido utilizadas como plastificantes en la obtención de *films*. Todos ellos se caracterizan por tener al menos un grupo hidroxilo libre y un grupo ácido, lo que les confiere la posibilidad de formar enlaces de hidrógeno con otras moléculas [192]. Como ocurre en el caso de los polioles, el tamaño de la molécula y el número de grupos hidroxilos presentes en la misma van a tener una gran influencia sobre las propiedades mecánicas de los *films* obtenidos.

El ácido láctico es un ácido orgánico de pequeño tamaño, de carácter predominantemente hidrófilo. Su estructura únicamente presenta un grupo ácido y un grupo hidroxilo libre. Puede ser utilizado como plastificante de otros polímeros o también es posible obtener *films* basados en el polímero resultante de la polimerización de esta molécula; el ácido poliláctico [193]. Sin embargo, hasta la fecha ha sido principalmente aplicado para la obtención de *films* destinados a otros fines distintos de su administración vaginal.

5.2.3. Ácidos grasos: ácido oleico

Los ácidos grasos son un grupo de moléculas anfifílicas que pueden ser utilizados como plastificantes de *films*. Su principal ventaja es que, en función de la hidrofilia del polímero con el que vayan a interactuar, pueden modificar su disposición para orientar su parte polar o apolar hacia la interfaz polímero-ácido [194]. Es por ello que son especialmente útiles a la hora de plastificar polímeros de naturaleza anfifílica. Los ácidos grasos utilizados con más frecuencia en la obtención de *films* son los ácidos oleico, láurico, palmítico, esteárico y linoleico [164, 166]. Sin embargo, no han sido empleados hasta la fecha en la plastificación de *films* vaginales. Esto se debe posiblemente a que

prácticamente la totalidad de los *films* diseñados para esta vía de administración están basados en polímeros hidrófilos, que resultan más compatibles con plastificantes polares.

El ácido oleico es un ácido graso, y como tal tiene un carácter anfifílico – presenta una cabeza polar unida a una cola apolar –. Dada la utilización de la zeína para la obtención de *films* vaginales, y el carácter anfifílico de dicha proteína, se utilizará el ácido oleico como plastificante. Estos dos compuestos han demostrado una excelente compatibilidad, dando lugar a una estructura muy característica denominada "sándwich proteína-lípido", en la que se alternan capas de ácido oleico y zeína y se consigue reducir notablemente la hidrofilia del *film* [195, 196].

5.2.4. Ésteres de ácidos orgánicos: trietilcitrato y tributilcitrato

Los ésteres de ácidos orgánicos – citratos, tartratos, sebacatos, etc. – son moléculas obtenidas por la esterificación de su correspondiente ácido orgánico – ácido cítrico, ácido tartárico, ácido sebácico, etc. – [164]. La adición de grupos éster donde originalmente se encontraban los grupos hidroxilo hace que la molécula se vuelva predominantemente hidrófoba, a pesar de que aún puede mantener algún grupo hidroxilo sin esterificar. Su uso es prácticamente exclusivo de *films* formulados con polímeros que tienen un marcado carácter hidrófobo, y esta es la principal razón de que no sean empleados para *films* de administración vaginal.

En esta tesis se utilizarán como plastificantes dos de los ésteres obtenidos a partir del ácido cítrico. Uno de ellos es el trietilcitrato, en el que predomina su carácter hidrófobo – aunque aún dispone de algún grupo hidroxilo libre –. El otro es el tributilcitrato, en el que la única diferencia con el trietilcitrato radica en que la esterificación se forma con un grupo butilo en lugar de con un grupo etilo. Aunque sus propiedades son muy similares, el tributilcitrato es ligeramente más hidrófobo por el mayor tamaño de las cadenas del éster.

5.3. Agentes solubilizantes incorporados en los geles desarrollados

Dada la poca solubilidad acuosa presentada por la dapivirina, uno de los fármacos empleados en esta tesis, fue necesario explorar la utilización de agentes solubilizantes para incrementar la solubilidad del principio activo en el medio vaginal. Las dos estrategias evaluadas son la adición de tensioactivos y la formación de complejos de inclusión con ciclodextrinas.

5.3.1. Tensioactivos: lauril sulfato sódico (LSS) y polisorbato 60

Los tensioactivos o surfactantes son moléculas de tamaño variable que se caracterizan por tener dos zonas diferenciadas en su estructura; una de naturaleza polar y otra apolar [197]. Existen distintos tipos de surfactantes, que frecuentemente son clasificados en iónicos o no iónicos en función de si se disocian en iones o no. Además, entre los iónicos es posible encontrar surfactantes aniónicos – el grupo hidrofílico se disocia en aniones cuando se encuentra en disolución –, catiónicos – se disocia en cationes – o anfóteros – se puede disociar en aniones o cationes en función del pH del medio –. Cuando se encuentran en disolución son capaces de formar micelas, estructura que permite que los tensioactivos puedan ser utilizados como estrategia para aumentar la solubilidad acuosa de fármacos poco hidrosolubles [197, 198]. En disolución, el fármaco hidrófobo quedaría retenido en el interior de las micelas, que orientan sus grupos funcionales polares hacia el exterior [199].

Para lograr la solubilización de la dapivirina, en esta tesis han sido empleados dos surfactantes, el LSS y el polisorbato 60. El LSS es un surfactante de naturaleza aniónica ampliamente utilizado por las industrias farmacéutica y cosmética [200]. Este tensioactivo ha sido incluido en formulaciones vaginales para mejorar sus propiedades, como por ejemplo en geles con la finalidad de modificar su viscosidad o dispersar el principio activo incluido [201, 202]. Pero también debemos destacar que el propio LSS ha sido evaluado como posible microbicida, ya que podría causar la disrupción de la membrana externa de algunos virus de transmisión sexual, tales como el VIH [203].

Por otro lado, el polisorbato 60 – también conocido como Tween® 60, el nombre con el que fue originalmente comercializado – es un surfactante no iónico. Se obtiene

mediante la esterificación de sorbitano con ácido esteárico. Los surfactantes no iónicos presentan la ventaja de ser menos tóxicos que los iónicos, lo que facilita su uso en aplicaciones biomédicas. Al ser reconocidos como inocuos han sido ampliamente empleados para mejorar la solubilidad de fármacos [204]. La vía vaginal no es una excepción, pues también encontramos varias formulaciones en las que se han utilizado los polisorbatos como excipientes [205-208].

5.3.2. Ciclodextrinas: 2-hidroxipropil-β-ciclodextrina

Las ciclodextrinas son moléculas cíclicas formadas por un número determinado de D-(+)-glucopiranosas que se unen a través de enlaces α-(1,4)-glucosídicos. Su conformación estructural se asemeja a un tronco de cono hueco, cuyo exterior – donde predominan grupos hidroxilos – es notablemente más hidrofílico que el interior [209].

El tamaño de la ciclodextrina va a venir determinado por el número de unidades de glucopiranosa que la forman. Así, las moléculas de α-ciclodextrina, β-ciclodextrina y γ-ciclodextrina se componen de 6, 7 y 8 unidades de glucopiranosa, respectivamente [210]. Cuantas más unidades forman la molécula, mayor será el diámetro de la cavidad interior de la ciclodextrina, manteniéndose la misma altura en los tres tipos [211].

La ciclodextrina empleada en el desarrollo de esta tesis es la 2-hidroxipropil-β-ciclodextrina. Se trata de un excipiente seguro, que se encuentra aprobado para su administración humana tanto por vía oral como tópica o intravenosa [212]. Al ser una β-ciclodextrina está constituida por siete unidades de glucopiranosa, en las que además algunos de los grupos hidroxilos libres han sido sustituidos por grupos hidroxipropilos. La principal ventaja que presentan tras esta sustitución es su mayor solubilidad acuosa. Aunque resulta sorprendente, pues estructuralmente la molécula es menos hidrófila al sustituir los grupos hidroxilos, este aumento de la solubilidad ha sido asociado a la desestabilización del estado cristalino de la molécula debido a la voluminosa sustitución [213]. Otra ventaja es la menor formación de agregados de ciclodextrinas a bajas concentraciones [214].

La principal utilidad de las ciclodextrinas se basa en su capacidad para incorporar fármacos lipófilos – o algún resto lipófilo de la molécula – en el interior de su cavidad,

formando complejos de inclusión con la misma [153]. Numerosas interacciones son responsables de dicha unión, pudiendo intervenir enlaces de hidrógeno, interacciones de Van der Waals, interacciones electrostáticas e interacciones hidrofóbicas [215]. Este complejo solamente puede formarse cuando las moléculas se encuentran en soluciones acuosas. Se produce por tanto un equilibrio entre las moléculas de fármaco libres en la disolución y las que se encuentran formando el complejo de inclusión con las ciclodextrinas [216].

La aplicación más destacada de la formación de complejos de inclusión entre ciclodextrinas y fármacos es la mejora de la solubilidad de estos últimos. Además, es posible utilizar ciclodextrinas con el fin de estabilizar o proteger al fármaco frente a la degradación, aislar compuestos incompatibles e incluso lograr la liberación controlada de principios activos [215, 217]. La inclusión de ciclodextrinas en formulaciones vaginales es bastante frecuente, pudiendo encontrar geles [218, 219], cremas [220] o comprimidos [108] con este excipiente.

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OBJETIVO Y PLANTEAMIENTO

El objetivo de esta tesis doctoral es el desarrollo de formulaciones de administración vaginal, con el fin de evaluar distintas estrategias que pudiesen ser aplicadas en el desarrollo de un microbicida vaginal con potencial para proteger a la mujer frente a la transmisión sexual del virus de inmunodeficiencia humana (VIH), superando las dificultades encontradas en los microbicidas desarrollados hasta la fecha.

Para justificar este objetivo se antoja imprescindible explicar la necesidad de desarrollar un microbicida vaginal frente al VIH. La posibilidad de desarrollar una vacuna que proteja frente a la transmisión del virus no parece factible a corto plazo. Sin ir más lejos, en febrero de 2020 fue paralizado el último ensayo clínico con una vacuna para este fin al no demostrar ninguna eficacia protectora. Es por ello que, mientras no exista una vacuna efectiva que permita inmunizar frente a la infección por VIH, el desarrollo de microbicidas vaginales se convierte en una estrategia fundamental para otorgar a la mujer en los países menos desarrollados poder de decisión y que dispongan de herramientas para la protección frente a la transmisión sexual del virus sin necesidad de cooperación del hombre.

La primera fase de este trabajo consistió en una revisión bibliográfica del desarrollo de los microbicidas frente al VIH a lo largo de las últimas décadas, para así conocer todos los éxitos y fracasos y poder valorarlos de cara al desarrollo de las formulaciones que compondrían esta tesis.

En base a dicha revisión bibliográfica se tomó la decisión de comenzar la investigación práctica con el desarrollo de una formulación de liberación sostenida de tenofovir, puesto que los mejores resultados hasta la fecha se habían logrado con un gel que contenía este principio activo, pero su principal inconveniente era que el microbicida desarrollado requería una administración diaria. Además, considerando el bajo poder adquisitivo medio de la zona en la que estos microbicidas tendrían mayor utilidad (África subsahariana), se optó por los comprimidos vaginales como la forma farmacéutica de elección para el desarrollo de este microbicida de liberación sostenida, ya que los comprimidos son no solo la forma farmacéutica más habitual en el mercado, sino también la más económica. La primera estrategia abordada fue la fabricación de comprimidos basados en polímeros con capacidad de gelificar en medio acuoso. De este

modo, tras su administración el comprimido absorbería el fluido vaginal para formar un gel, con la intención de que dicho proceso de gelificación pudiese conseguir controlar la liberación del fármaco antirretroviral y en consecuencia lograr una formulación de liberación sostenida. Otra característica exigida a estos comprimidos fue su capacidad mucoadhesiva, para así garantizar la retención de la forma farmacéutica en el medio vaginal durante el tiempo necesario para conseguir la liberación del fármaco. Para ello, en primer lugar se prepararon comprimidos vaginales con distintos polímeros por separado, con el fin de evaluar sus propiedades y su potencial para el desarrollo de microbicidas de liberación sostenida de fármacos. La siguiente etapa consistió en la combinación de distintos polímeros, con el objetivo de aunar las ventajas que cada uno de ellos pueden ofrecer. Finalmente, se evaluó la posibilidad de granular el fármaco junto con sustancias hidrófobas, que posteriormente serían incluidos en una matriz formada por los polímeros gelificantes evaluados anteriormente, buscando así un mecanismo combinado de control de la liberación del fármaco que permitiera espaciar aún más la frecuencia de administración requerida.

La siguiente etapa del trabajo consistió en desarrollar esta misma estrategia de liberación sostenida de tenofovir, pero desde una formulación alternativa como son los *films* vaginales. Debido a que se trata de una formulación mucho menos estudiada, el primer paso consistió en realizar una revisión bibliográfica con el fin de adquirir conocimiento sobre las materias primas requeridas para la elaboración de los *films* – polímeros y plastificantes, principalmente –, las técnicas de fabricación disponibles – con especial atención a los parámetros que deben ser controlados en cada una de ellas – y los ensayos de caracterización que son más frecuentemente realizados por los investigadores en este campo de estudio. Para el desarrollo de los *films* vaginales de liberación sostenida de tenofovir se evaluaron dos polímeros con potencial para la liberación sostenida de fármacos, pero con muy distintas características en términos de hidrofilia/hidrofobia. Su combinación, así como la modificación de sus propiedades al incorporar distintos agentes plastificantes, también fue evaluada.

A continuación, se decidió evaluar la posibilidad de desarrollar formulaciones microbicidas inteligentes, en las que la liberación del fármaco se realizase de forma pH-dependiente – viéndose por tanto acelerada en el momento en que entra en

contacto con el fluido seminal, mucho más alcalino que el fluido vaginal –. Una primera estrategia consistió en, partiendo de la base de los *films* ya desarrollados para la liberación sostenida del antirretroviral, realizar modificaciones en cuanto a los plastificantes utilizados – naturaleza de los mismos, combinación de distintas moléculas, proporción con respecto al polímero, etc. – para así conseguir una degradación más rápida del *film* en presencia del fluido seminal que conllevase una liberación más rápida del fármaco en este pH. Posteriormente, esta misma estrategia de la preparación de *films* con liberación pH-dependiente de tenfovir fue desarrollada de una manera diferente; se evaluó el potencial de distintos polímeros para controlar la liberación del fármaco en fluido vaginal y, debido a un cambio de solubilidad o de permeabilidad cuando el medio es alcalinizado, liberar de forma rápida el antirretroviral en presencia del fluido seminal.

La etapa final de este trabajo se centró en el desarrollo de una forma farmacéutica vaginal alternativa – denominada discos vaginales – consistente en geles liofilizados con forma plana, asemejándose a la forma de los *films*, para así ofrecer mayor comodidad y facilidad de administración. Con estos liofilizados se evaluó una tercera estrategia de protección frente a la infección; el desarrollo de microbicidas de administración coito-dependiente que permitieran una rápida liberación de fármacos antirretrovirales tras su administración, para así conseguir concentraciones inhibitorias en el medio vaginal en poco tiempo. Además, conjuntamente se evaluó la posibilidad de incorporar en las formulaciones distintos agentes que mejorasen la solubilidad del principio activo, para que estos discos vaginales pudiesen permitir una rápida liberación no solo de tenofovir sino también de dapivirina – un antirretroviral menos hidrosoluble –.

RESULTADOS DE LA INVESTIGACIÓN

CAPÍTULO I

HISTORICAL DEVELOPMENT OF VAGINAL MICROBICIDES TO PREVENT SEXUAL TRANSMISSION OF HIV IN WOMEN: FROM PAST FAILURES TO FUTURE HOPES

Drug Design, Development and Therapy

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REVIEW

Historical development of vaginal microbicides to prevent sexual transmission of HIV in women: from past failures to future hopes

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Abstract: Infection with human immunodeficiency virus (HIV) remains a global public health concern and is particularly serious in low- and middle-income countries. Widespread sexual violence and poverty, among other factors, increase the risk of infection in women, while currently available prevention methods are outside the control of most. This has driven the study of vaginal microbicides to prevent sexual transmission of HIV from men to women in recent decades. The first microbicides evaluated were formulated as gels for daily use and contained different substances such as surfactants, acidifiers and monoclonal antibodies, which failed to demonstrate efficacy in clinical trials. A gel containing the reverse transcriptase inhibitor tenofovir showed protective efficacy in women. However, the lack of adherence by patients led to the search for dosage forms capable of releasing the active principle for longer periods, and hence to the emergence of the vaginal ring loaded with dapivirine, which requires a monthly application and is able to reduce the sexual transmission of HIV. The future of vaginal microbicides will feature the use of alternative dosage forms, nanosystems for drug release and probiotics, which have emerged as potential microbicides but are still in the early stages of development. Protecting women with vaginal microbicide formulations would, therefore, be a valuable tool for avoiding sexual transmission of HIV.

Keywords: vaginal formulations, microbicides, prevention, sexual transmission, acquired immunodeficiency syndrome (AIDS), human immunodeficiency virus (HIV)

Introduction

Acquired immunodeficiency syndrome (AIDS) is a global health concern. It is a chronic infectious disease caused by the human immunodeficiency virus (HIV), an enveloped virus in the Retroviridae family with a lipid membrane and is capable of interacting with $CD4^+$ T cells, thanks to gp120. The virus infects the cells of the immune system, destroying or impairing their function and causing a progressive deterioration of the immune system until the sufferer falls into an immunodeficient state. Primary HIV infection is symptomatic in over half the reported cases, but may be overlooked as the symptoms resemble those of a common viral infection. There are two serotypes of the virus (HIV-1 and HIV-2), whose key difference in functional terms is that in HIV-2 infection, the amount of circulating viruses is lower than in HIV-1 infection, making its evolution slower and incubation period longer. Nevertheless, both serotypes ultimately cause AIDS.[1] AIDS is the most advanced stage of HIV infection, in which the immune system ceases to respond effectively and diseases develop due to the loss of the body's defense capability.[2]

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Table 1 Classification of antiretroviral drugs

Mechanism of action	Drugs
Entry inhibitors or FIs	Enfuvirtide, maraviroc
NRTIs	Tenofovir, adefovir, zidovudine, didanosine, stavudine, emtricitabine, abacavir, lamivudine
NNRTIs	Efavirenz, rilpivirine, nevirapine, dapivirine or etravirine
PIs	Ritonavir, darunavir
IIs	Dolutegravir, raltegravir

Abbreviations: FIs, fusion inhibitors; IIs, integrase inhibitors; NRTIs, nucleoside reverse transcriptase inhibitors; NNRTIs, non-nucleoside reverse transcriptase inhibitors; PIs, protease inhibitors.

The goal of ending the AIDS epidemic is becoming more attainable through the elimination of HIV transmission and AIDS-related deaths,[3,4] which is now possible thanks to the numerous antiretroviral drugs with different mechanisms of action currently available for the treatment of HIV (Table 1). These drugs are able to inactivate the virus at different stages of the viral cycle (Figure 1).

However, stigma and discrimination, violence against women and girls and unjust laws continue to hamper efforts to achieve global targets. If these challenges can be overcome, and if efforts to prevent HIV continue to gain in efficacy – and with the rapid rise in the number of people receiving treatment – the world will attain its goal of ending the AIDS epidemic in the not-too-distant future.

The seventh objective set by the United Nations for bringing an end to AIDS specifically raises the question of the current status of women – particularly in low- and middle-income countries – in terms of protection against HIV, since the likelihood of transmission of sexually transmitted infections (STIs) from men to women has been observed to be alarmingly high.[5]

This is because current methods of preventing STIs, such as abstinence, condoms and monogamy, are often ineffective and outside the control of women; many men oppose the use of condoms and women do not have the authority to insist that their partners use them. Thus, today, over 50% of new HIV infections are in women.

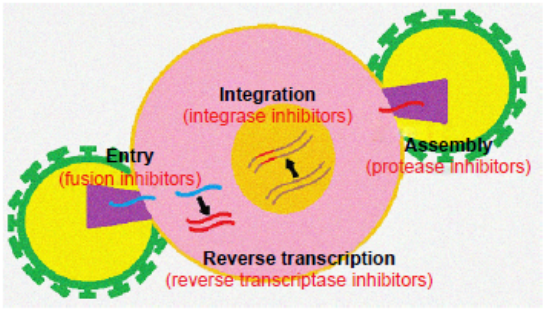

Figure 1 Targeting site of antiretroviral drugs at different stages of the viral cycle.

Sexual behavior is more likely to be the cause of STI transmission in certain countries, and there are indications that unsafe sex has increased in several nations. The latest data point to a significant rise in polygamy in a number of countries (Burkina Faso, Congo, Ivory Coast, Ethiopia, Gabon, Guyana, Rwanda, South Africa, Uganda, Tanzania and Zimbabwe) as well as a decline in condom use (Ivory Coast, Niger, Senegal and Uganda).[5]

The main setbacks to efforts to prevent HIV transmission are the lack of access to sexual education services and sexual violence against young women and girls. Each year, about 380,000 women aged between 10 and 24 years suffer from HIV infection, meaning that 50 young women become infected with HIV every hour. Furthermore, 80% of women aged 10–24 with HIV live in sub-Saharan Africa.[6]

It is, therefore, necessary to have female-controlled methods such as microbicides that may be used to prevent vaginal acquisition of HIV.

Microbicides are currently seen as a promising tool to protect women from acquiring this type of infection. The use of microbicides is be controlled by women, who can apply them before intercourse without the man's cooperation. A vaginal microbicide can be defined as any agent included in a topical formulation designed to prevent the spread of sexually transmitted pathogens either through cell death, inactivation of cell mechanisms, inhibition of viral replication, the formation of a physical barrier between cells and pathogens, or by enhancing the natural protection mechanisms of the cervix and vagina.[7]

Unfortunately, many vaginal microbicide formulations may fail to produce a protective effect due to their lack of efficacy and their unsuitable formulation.[8] Some of the most frequently used vaginal dosage formulations include creams, gels,[9–13] tablets,[14] films[15] and intravaginal rings,[16–18] each of which has particular advantages and drawbacks.

In view of the above, there is little doubt that the development of a safe, effective and affordable vaginal microbicide that is easy to manufacture, stable under different environmental conditions and comfortable for women to administer themselves would represent a major breakthrough in preventing HIV transmission.

Historical development of vaginal microbicides

In recent decades, very different strategies have arisen to prevent the sexual transmission of HIV. According to their mechanism of action, we can distinguish microbicides

without antiretroviral drugs, such as surfactants, polyanions, acidifiers and gp120 neutralizing monoclonal antibodies, and microbicides-containing drugs used for HIV treatment, for example, entry inhibitors or inhibitors of viral enzymes. These microbicides can inhibit virus transmission at different sites: while the virus is in the vaginal environment, it can be inactivated by microbicides containing surfactants, polyanions or monoclonal antibodies, or due to acidic pH achieved with the use of acidifiers; or, once HIV has passed through the vaginal epithelium, it can be prevented from internalizing in $CD4^+$ T cells by entry inhibitors, or else, viral replication can be prevented with reverse transcriptase inhibitors (Figure 2). However, after years of research with different potential microbicide substances, the current trend is toward the development of microbicides with antiretroviral drugs such as maraviroc (MVC) (ViiV Healthcare, Brentford, United Kingdom), tenofovir (TFV) (Gilead Sciences, Cambridge, United Kingdom) or dapivirine (DPV) (IPM, Silver Spring, MD, USA), which have offered the most promising results in clinical trials.

First attempts to prevent sexual transmission of HIV: vaginal gels

Gels are possibly the most widely studied pharmaceutical formulations for developing vaginal microbicides. They were the pharmaceutical dosage form of choice for the first vaginal microbicides, possibly because they have the advantage of being easily and conveniently applicable by women, which makes the use of such formulations greatly improve adherence to treatment.[19] In addition, their manufacturing cost is not very high, especially compared to more sophisticated forms, and they are easy to mass produce. Gels are optimal formulations for ensuring the microbicide begins to exert its action quickly; however, they are generally unable to retain the drug and provide sustained release over time. They also require certain conditions of conservation, as they are not particularly stable against adverse environmental conditions.

Surfactants, the first failure

Surfactants such as nonoxynol-9 or Savvy gel[80] were the first substances evaluated as microbicides. They act as a virucide by lowering the surface tension of the pathogen, resulting in the death of the microorganism before it comes into contact with the vaginal mucosa.[20,21] However, the lack of effective protection for women highlighted by the studies led to the rapid rejection of the use of surfactants in microbicide formulations.[20–23] This was not all; tests showed that not only is nonoxynol-9 ineffective in preventing HIV transmission, but also it increases the incidence of genital lesions such as vaginal ulcers, thus raising the risk of STIs.[20,24]

Screening of alternative substances: acidifiers, polyanions, monoclonal antibodies and entry inhibitors

After the failure of surfactants, new strategies to develop vaginal microbicides turned toward mechanisms that work by preventing the pathogen from entering the cells. The most

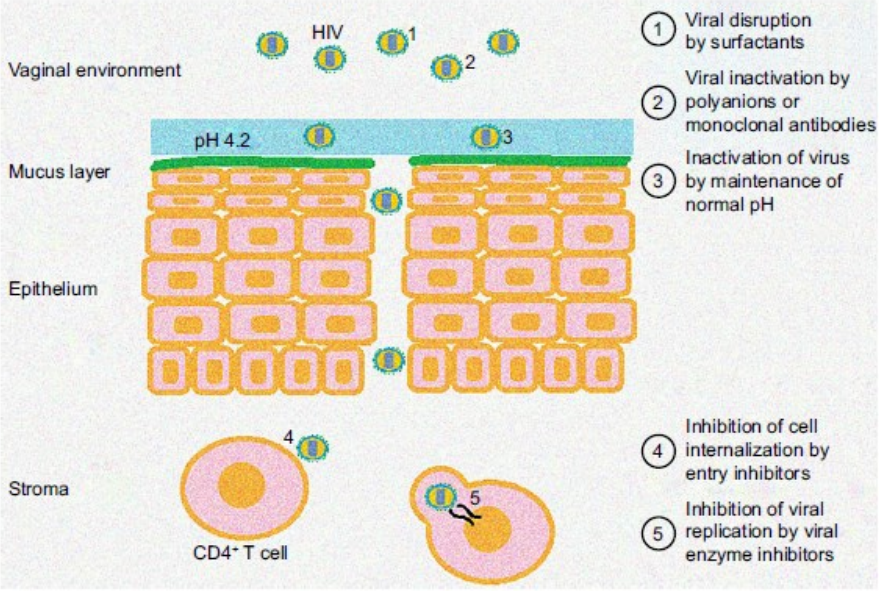

Figure 2 Diagram of the action sites of different microbicides against HIV.

basic formulations simply prevent contact between the surface of the vaginal mucosa and pathogens.

Carraguard® is a gel under research whose active ingredient is carrageenan, a sulfated linear polysaccharide extracted from seaweed. The microbicide was formulated as a gel to be applied to the vaginal mucosa in the hour prior to sexual intercourse.[25] Studies have shown that its use is safe for women, and the associated side effects are mild and infrequent.[26,27] This gel also proved its ability to reduce the risk of sexual transmission of human papillomavirus.[28] However, Phase II trials failed to show efficacy in preventing HIV transmission.[25,29,30] The use of the gel is currently being assessed as a carrier of an antiretroviral drug.[25]

Another mechanism designed to prevent the entry of pathogens into the cells was the use of acidifiers such as BufferGel®. This acidifier acts as a buffer and is capable of maintaining normal vaginal acidity in the presence of ejaculated semen. This microbicide is based on studies suggesting that an acidic environment could inhibit HIV.[31] However, although studies on BufferGel have shown it to be safe, it has not demonstrated effectiveness in preventing HIV transmission.[32,33]

The lack of efficacy of these strategies prompted the search for a very diverse range of substances capable of interacting directly with the virus,[34] from polyanions or monoclonal antibodies able to recognize the HIV and bind to it to antiretroviral drugs that act as fusion inhibitors, such as MVC.

One example of the gels tested as a means of blocking the pathogen is PRO 2000, whose active ingredient is the synthetic polymer naphthalene sulfonate. This is a negatively charged polyanion capable of interacting with the positive charges of viral gp120 to block virus entry into cells.[35] It has proven antiviral activity against HIV-1 in vitro and in animal models, and a favorable risk profile.[33,36,37] Nevertheless, although trials in women have demonstrated high adherence and safety, it has no demonstrated effectiveness in preventing sexual transmission of HIV.[33,37]

VivaGel® is another polyanion currently under study, whose active ingredient is SPL7013, a dendrimer expressly created using nanotechnology to show antiviral activity against HIV,[38] and is formulated in a mucoadhesive carbopol gel.[39] It has shown antiviral activity against HIV in the presence of seminal plasma,[40] as well as in animal models,[38,41] along with good tolerance in animals and humans.[38,42–44] However, subsequent clinical trials revealed adverse effects associated with this formulation, leading to its rejection on the grounds of being unsafe for continued use in women.[45,46]

A further study confirmed the inefficacy of polyanions in clinical trials despite their in vitro efficacy. This ineffectiveness was due to the formation of a semen-derived enhancer of virus infection, which promoted HIV infection in the presence of semen.[47,48] Although Sonza et al reported that this enhancement effect is not applicable to all polyanions,[49] it highlighted the importance of performing the effectiveness tests in the presence of semen.

Some microbicidal formulations also include gp120-neutralizing monoclonal antibodies, which recognize and bind to the viral gp120, thus preventing the virus from binding to $CD4^+$ T lymphocytes. Examples include vitamin B_{12} and the monoclonal antibodies 2G12 and PRO-140.[50–53]

Other agents with the ability to bind to gp120 are lectins.[54] The lectin most widely studied for its ability to bind HIV is probably CV-N, a protein from cyanobacteria capable of potently inactivating HIV-1, HIV-2 and simian immunodeficiency virus by binding irreversibly to gp120. Research into this active ingredient has proved its effectiveness in blocking infection by HIV-1 in ectocervical human explants.[55] It was formulated in 1% and 2% CV-N gels, and in all cases showed protection against the virus in monkeys without any adverse or cytotoxic effect.[56] CV-N could, therefore, be a good candidate for human trials as a topical microbicide against HIV. In order to reduce the high cost of its production, efforts have been made to develop transgenic plants capable of producing CV-N, achieving positive results in rice endosperm, *Nicotiana tabacum* and soybean seeds.[57–59] Griffithsin, a protein from red seaweeds, is another drug with the same mechanism of action. Studies to date are encouraging, as they have demonstrated its activity in picomolar concentrations and absence of irritability and inflammation.[60,61] It has also been successfully expressed and purified from transgenic plants, which would make it more economical.[60,62–64] Although it has yet to be tested in animals, it already has proven features that represent a significant step forward in the development of vaginal microbicides based on gp120-neutralizing monoclonal antibodies, as it would solve the main problems found during experimentation with other active ingredients in this family, namely, the need for high concentrations and the high production costs.[60,62]

Finally, it is worth mentioning microbicides that contain entry inhibitors such as MVC, an approved antiretroviral drug for treatment of HIV-1 CCR5-tropic in adults.[65] It is specifically an antagonist of the CCR5 receptor, a protein located in T lymphocytes, which binds to HIV at the time of cell entry. MVC binds to these receptors and prevents HIV

from infecting cells and multiplying. However, this drug is not active in all patients, as in some subjects, the virus uses another receptor called CXCR4 to enter the cells.[66]

There are several studies aimed at testing the efficacy of MVC as a microbicide, which have proven its efficacy even in the presence of semen[48,67] and its tendency to concentrate in the cervicovaginal fluid and vaginal tissue.[68] One of these trials assessed the efficacy of a topical vaginal gel of hydroxyethyl cellulose containing 2.2% MVC in humanized mouse strains (RAG-hu). The gel was applied to female mice before exposing them vaginally 1 hour later to HIV-1, in order to compare its protective efficacy versus the placebo gel; no mouse was infected with the virus, while those treated with the placebo were.[69]

Another trial with this active ingredient studied the effectiveness of a hydroxyethyl cellulose gel with different concentrations of MVC in macaques, where it was found that complete protection was achieved against the virus with the gel with 3.3% MVC. However, this protection could only be achieved with a high concentration in the vaginal fluid, which was only attained between 30 minutes and 2 hours after the administration of the gel. Protection, therefore, largely depends on the time elapsed between application and contact with the virus.[70,71]

Following these assays, several studies have sought to lengthen the residence time of the gel. One proposed formulation tested in macaques was a silicone gel with MVC[72] compared to a hydroxyethyl cellulose gel with the same load of MVC, which achieved greater and more sustained MVC concentrations in the vaginal fluid.[73]

Inclusion of viral enzyme inhibitors: first positive results

Although, as we have seen, the initial trend in the search for an effective microbicide was to use substances to prevent the entry of the virus in order to block the first step of infection,[74] after unsuccessfully evaluating numerous compounds in clinical trials, the focus switched to the study of potential microbicides with antiretroviral drugs that prevent virus replication.[75]

Nucleoside reverse transcriptase inhibitors (NRTIs) were the first antiretroviral drugs to show activity against HIV. Several drugs in this family are used today to treat HIV patients and are being studied as potential microbicides. Possibly, the most widely studied drug in this group is TFV, which acts in the case of HIV infection by blocking the activity of reverse transcriptase and preventing the virus from infecting cells and replicating.[76,77]

Although the antiretroviral activity of TFV has been confirmed and it has been approved for oral use, research is required into its potential microbicidal effect against HIV, in addition to efficacy and safety studies, before vaginal pharmaceutical forms with this active ingredient can be developed. TFV microbicide formulations have proven antiviral efficacy in vitro[78] and in animal models,[79,80] and have been evaluated in Phase III clinical trials. Studies with a TFV-based vaginal gel have shown it has no significant cytotoxicity in women.[81–83] Finally, several safety studies with this microbicide indicate that TFV has no toxicity for vaginal mucosa at concentrations commonly used as a microbicide.[11,18,84] It has also proven to be acceptable and well tolerated by women.[81,85,86]

The CAPRISA 004 study evaluated the effectiveness of a 1% TFV gel in preventing HIV transmission in South African women.[87] The gel was found to reduce HIV infection by 39% and even 54% when women had high adherence to treatment.[87,88]

Following the successful CAPRISA 004 study, numerous further projects sought to reproduce the TFV gel. The MTN-001 study compared the use of a TFV gel with the oral administration of this drug, and found that higher concentrations of the drug could be achieved in the vaginal tissue with vaginal administration.[89] The same study also demonstrated lower adherence to microbicides in sub-Saharan Africa compared to the USA.[90] More recent studies have confirmed the effectiveness of the gel;[91] but in the VOICE trial, using a 1% TFV gel in African women was not observed to reduce the likelihood of HIV infection, although adherence to treatment was low.[10]

Although the recent negative results of some tests are puzzling,[91] evidence supports the efficacy of this antiretroviral in preventing the transmission of HIV-1,[88,92,93] offering a cost-effective method that can be controlled by women.[94,95] The success of the TFV gel was a milestone in the development of an effective vaginal microbicide to prevent sexual transmission of HIV.[93,96]

However, the clinical trials with this gel also served to highlight the importance of analyzing the different parameters that could influence its efficacy.[96] According to the results of the clinical trials, adherence is the key factor in achieving protection.[97–100] Other factors influencing its effectiveness include adhesion of the formulation to the mucosa, which could alter the drug concentration and the integrity of the vaginal mucosa,[97] and the time elapsed between gel application and sexual intercourse.[101] Another factor that has been shown to be important in the efficacy of the

formulation is systemic innate immune activation prior to infection; one possible mechanism to increase the efficacy of the TFV gel is the addition of a suppressor of innate immune system activation.[102]

Another family of antiretroviral drugs consists of non-nucleoside reverse transcriptase inhibitors (NNRTIs). These are noncompetitive inhibitors that bind to an allosteric site on the reverse transcriptase and induce conformational changes in the enzyme.[103] Various NNRTIs have been studied as microbicides, including DPV and rilpivirine,[104–106] and multiple NNRTIs have been specifically designed with the ability to bind more closely to HIV-1 retrotranscriptase, such as MIV-150, a derivate of urea–phenethylthiazolylthiourea,[65,103] and UC78, a thiocarboxanilide.[103]

Several studies have been carried out in macaques using different pharmaceutical forms containing MIV-150, an example of which is the combination of MIV-150 with Carraguard.[107] As previously described, Carraguard is a carrageenan gel that showed no efficacy in clinical trials, but has been assessed as a vehicle for antiretroviral drugs in the development of vaginal microbicides.[25] In vitro assays demonstrated greater antiretroviral activity of the MIV-150/carrageenan combination compared to Carraguard, and this efficacy was not modified by the presence of seminal fluid.[107] However, in vivo assays with this combination ruled out its potential as a microbicide due to the predominance of the barrier effect of Carraguard, which prevented the combination with MIV-150 from being more effective.[108] Nevertheless, the MIV-150 in the placebo gel limited vaginal infection, confirming the potential of topical NNRTIs in preventing sexual transmission of HIV.[108] Another gel combining MIV-150 and zinc acetate showed protection in animal models against simian–human immunodeficiency virus (SHIV) for 24 hours after vaginal administration, improving the protection achieved when the gel contained only one of the agents.[109,110] The MIV-150/zinc acetate/Carraguard combination (MZC) was also evaluated, and was found to provide significant protection in macaques with its pre- and postcoital application.[110–114] An in vitro study comparing the efficacy of MZC gel versus 1% TFV gel showed increased activity against SHIV-retrotranscriptase with MZC gel.[115] After the promising data shown by this formulation, the first clinical trials were eagerly awaited. The results of the first clinical trial with MZC gel have recently been published, showing that the gel is safe and well tolerated by women.[116]

DPV is a much studied drug in this group, and has undergone clinical trials as one of the most promising drugs for the development of microbicides to prevent HIV-1 transmission. The studies have demonstrated its in vitro activity to prevent infection even in the presence of semen,[117] and further trials in animal models showed that DPV does not irritate the vagina.[118] Gels containing this antiretroviral were formulated and evaluated in animal models, and a high concentration of DPV was found in the vaginal tissue after administration.[119] The first clinical trials with DPV gel were subsequently performed, demonstrating that its administration is safe and well tolerated by both women[120–122] and men.[123] Another finding was that much higher concentrations were achieved with the vaginal release of DPV from the gel than were required to achieve HIV inhibition in vitro.[121]

However, the effectiveness of drugs from this family must be evaluated in women, and especially in continuous prophylactic use, as their main drawback is the rapid development of HIV resistance to these drugs.[124]

Trend change in microbicide formulation: development of vaginal rings

Although the results obtained in recent years with gels containing reverse transcriptase inhibitors increase the hope of developing a microbicide that will significantly reduce HIV transmission, the creation of an effective vaginal microbicide also implies knowledge of the circumstances of the target population, and transmission prevention strategies must be adapted accordingly.[125] This is why much of the current effort focuses on understanding the aspects that govern the effectiveness of microbicides, among which a key factor is considered to be adherence in trials. Strategies must, therefore, be designed to improve adherence and the factors that influence it. These range from supporting the users, assessing their perception of risk and analyzing their social background and relationship problems, including whether or not their partners allow them to use microbicides. As a result, attention has shifted toward the development of formulations that require less commitment from the user to show efficacy, such as sustained drug release formulations.[126,127] Since adherence to treatment has been seen as a crucial factor, sustained-release formulations of antiretrovirals, such as vaginal rings, may lead to increased adherence, requiring less frequent application to achieve the necessary efficacy to avoid transmission.[127–129]

Vaginal rings could, therefore, represent a real alternative, as although they require a higher financial investment and are more complicated to manage, they have the advantage of allowing the sustained release of the drug over time periods of almost a month.[130,131] The increase in cost could be significantly offset by the decrease in the number of

applications and the subsequent improvement in adherence to treatment. The mass production of this dosage form is becoming increasingly advanced.

Since TFV has been the only vaginal microbicide to date to demonstrate protective efficacy in clinical trials, a wide range of formulations emerged that sought to improve the initial design in order to achieve effective microbicides that provide greater benefit than 1% TFV gel.[132]

Silicone[133] and polyurethane[134] rings containing TFV were formulated, and were able to release the drug for 25–30 days in the in vitro assays. In vivo assays with silicone rings on macaques demonstrated the safety of the formulation, as no adverse effects were observed, and they provided sustained release of TFV for >28 days.[135] The polyurethane-based rings were studied in sheep, where a good safety profile was also observed, with the drug release up to 90 days in this case.[132]

As an alternative formulation, vaginal rings were also developed with tenofovir disoproxil fumarate (TDF), a prodrug of TFV (Figure 3). The in vitro models showed that TDF can inhibit HIV at concentrations hundreds of times lower than TFV, and maintain its antiviral activity in the presence of semen.[136] The efficacy of intravaginal rings loaded with TDF was tested in female macaques versus placebo, where the rings showed complete protection against repeated exposure to the virus in the form of weekly exposure for 16 weeks.[137] In a similar study, macaques were exposed to the virus vaginally once a week for 12 weeks. All the monkeys treated with the placebo were infected, but only one of the six that received the TDF-loaded ring was infected.[17] These results indicate that the TDF ring provides lasting protection even against repeated exposure to the virus. Continuous-use trials of the formulation also showed that following continuous 6-month administration of these rings in macaques, the TFV levels in vaginal tissues and secretions remained constant and no adverse effects were observed.[16]

Given the excellent results in the animal tests, clinical trials were begun with TDF rings. It has recently emerged from the results of the Phase I trials that their use is safe and well tolerated by women, and that TFV concentrations in the vaginal mucosa from the ring are capable of offering protection against HIV.[138]

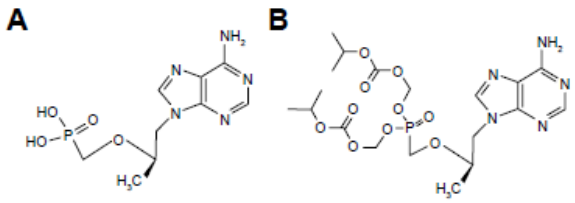

Figure 3 Chemical structure of tenofovir (**A**) and tenofovir disoproxil fumarate (**B**).

Leveraging the intravaginal ring as an excellent vehicle for multiple drug administration, the combination of TFV and acyclovir was also evaluated to prevent the transmission of the herpes simplex virus,[133] or with the addition of levonorgestrel to devise a ring that protected against HIV transmission and also had a contraceptive effect.[139]

TFV was not the only drug assessed for use in microbicidal vaginal rings. Another study on the same subject was designed to obtain sustained-release dosage forms of entry inhibitors. This involved pharmacokinetic studies with silicone elastomer vaginal rings containing MVC or CMPD167, two CCR5 receptor inhibitors, which release the active substances in a controlled manner over 28 days. The study showed that in macaques, this formulation could achieve vaginal fluid concentrations of above the inhibitory concentration in 50% of cases (IC_{50}) for both drugs, although the concentration of MVC was significantly higher than in the case of CMPD167.[140]

However, vaginal rings containing NNRTIs are the most widely studied for preventing the sexual transmission of HIV. The antiretroviral MIV-150 was initially the most commonly used, probably due to the results obtained with the gel.[141–143] An intravaginal ring loaded with MIV-150 demonstrated significant protection against SHIV infection in macaques.[141] As in the MIV-150 gel, the drug was subsequently evaluated in association with zinc acetate and carrageenan in order to improve the protection offered and reduce the dose of MIV-150, with a view to minimize its toxicity, the development of resistances and the cost of the formulation. These rings demonstrated their ex vivo effectiveness in monkey genital mucosa.[144]

Other NNRTIs evaluated for the development of vaginal rings are MIV-160,[145] UC781[146,147] and MC1220.[148] The efficacy of the vaginal ring with MIV-160 was compared with a carrageenan gel containing the same drug, but efficacy was observed only with the vaginal ring when the two dosage forms were assessed in vitro, highlighting the importance of choosing the appropriate formulation for the development of vaginal microbicides.[145] Poorer results were observed when evaluating UC781 in macaques, as no in vivo correlation was observed with the data obtained in vitro studies due to the poor solubility of the drug.[147] In the case of rings containing the drug MC1220, only partial protection against HIV infection was observed in studies with macaques.[148]

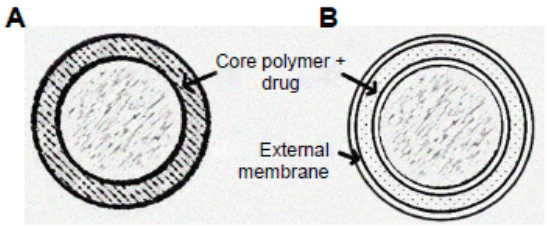

Figure 4 Structure of a matrix type (**A**) and reservoir type (**B**) vaginal ring.

Based on the results to date, undoubtedly, the most successful drug for use in vaginal rings to protect against sexual acquisition of HIV is another NNRTI, DPV. Vaginal rings were developed that were capable of releasing DPV in a sustained manner for 28–30 days. The materials used in their manufacture (polyurethane, silicone and others) and the type of ring (reservoir type or matrix type) were also assessed to obtain information about their impact on the effectiveness of the formulation (Figure 4).[149,150] Various safety trials were conducted in women with vaginal rings loaded with DPV, all of which showed safety and good tolerance, in addition to controlled drug release for 28 days, and the ability to obtain far greater DPV concentrations in the genital tract than in the case of IC_{50} against HIV-1.[151–154]

These excellent results led to the DPV rings undergoing Phase III clinical trials, the furthest stage for any microbicide containing an NNRTI. The ASPIRE Phase III study evaluated the efficacy of a silicone matrix ring containing DPV in African women.[155] Recent results from this study have shown that the DPV ring reduces HIV infection by 27%, and even up to 37%, excluding data from places where adherence was low. It was also noted that efficacy was much greater among women over 21, who also had greater adherence to ring use. Finally, it should be emphasized that there was neither an increase in adverse effects among users nor any development of resistance among the infected women.[156] Another Phase III study with a ring containing DPV, known as The Ring Study, found it reduced the risk of HIV acquisition by 31%. The results of this study coincide with the ASPIRE trial in claiming that the efficacy is much greater in women over 21, suggesting the influence of the physiology of the vaginal tract, a lower adherence to the use of the ring or the frequency of sexual intercourse as potential factors affecting efficacy. This study also coincides with the ASPIRE trial by ruling out the incidence of adverse effects and the development of resistances.[154]

These findings undoubtedly suggest that intravaginal ring delivery of DPV is a viable option for HIV prevention that merits further study, since as in clinical trials with the TFV gel, adherence was once again highlighted as a key factor in the efficacy of HIV prevention.[128,150,152,156,157]

Development of alternative dosage forms

While initially gels were the pharmaceutical form of choice for vaginal microbicides, and although in recent years vaginal rings have gained acceptance due to their ability to control release over long periods of time, these are not the only dosage forms that have been investigated for this purpose. This variety is necessary because the efficacy of the same antiretroviral drug varies depending on the formulation selected, and because the development of different pharmaceutical forms ensures that women have a wide range of options to protect themselves from the transmission of the virus, and therefore, each user can select the one that best suits her characteristics.[158,159]

Vaginal tablets

Tablets have the advantage of being easy and economical to manufacture on an industrial scale, easy to handle and stable under different environmental conditions.[7,159] If the aim is instant protection after administration, fast-dissolving tablets can be obtained depending on the excipients used in their development.[159,160] Tablets can also be manufactured to release the drug in a sustained manner, enabling controlled drug release and remaining effective for longer, in turn requiring fewer applications and favoring adherence to treatment. One example of this is the tablets made from polymers capable of gelling in the presence of vaginal fluid (Figure 5).

The first references to vaginal tablets to prevent sexual transmission of HIV were of Praneem polyherbal vaginal tablets. Their active ingredient was purified extract of *Azadirachta indica*, which had shown some in vitro activity against HIV.[161] Certain adverse effects such as genital itching or irritation were observed in Phase I clinical trials, but it was concluded that the use of the tablets daily for 14 days was safe and accepted by women.[161,162] Phase II clinical trials to evaluate their long-term safety concluded that their use for 6 months was equally safe and acceptable.[163,164] However, failures experienced by other formulations contemporary to these tablets, such as nonoxynol-9 or Carraguard gels, demonstrated the need for further preclinical evaluations to verify their effectiveness, and these tablets ultimately never underwent Phase III trials.

Years later, the idea of developing vaginal tablets for HIV prevention was revisited, with the key candidates

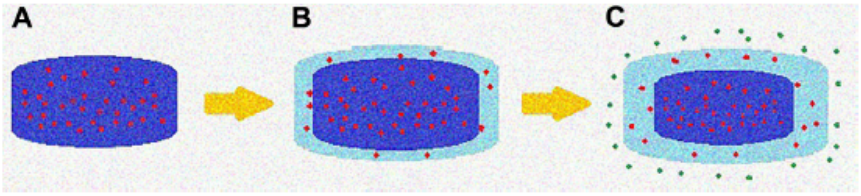

Figure 5 Mechanism of drug release from sustained release tablets by in situ gelation of the polymer.
Notes: At the time of administration, the tablet is completely solid (**A**). In contact with the vaginal fluid, the polymer of the outer layers forms a drug-loaded gel (**B**). The drug reaches the vaginal environment by diffusing through the gel layer or by erosion of the gel (**C**).

being the antiretroviral drugs that had proved most successful in other pharmaceutical forms. TFV vaginal tablets were found, alone or in combination with emtricitabine, another NRTI, to achieve similar concentrations of TFV in the vaginal environment to the 1% TFV gel, and proved effective in clinical trials.[14,76,165] Vaginal tablets[166] and lyophilized gels[167] containing DPV, the other drug shown to be effective in clinical trials, have also been developed.

However, these tablets were intended for daily application, or for use in a coitus-dependent manner in the case of immediate-release tablets, so the trend toward pharmaceutical forms capable of releasing the drug for several days led to the abandonment of these tablets.

More recently, we have seen the emergence of some alternatives such as osmotic tablets with IQP-058, which have shown an ability to achieve high levels of the drug in the vaginal mucosa for 10 days (Figure 6).[168] Lastly, current research is exploring the possibility of manufacturing controlled-release TFV vaginal tablets formulated with mucoadhesive polymers – such as carrageenan, chitosan or cellulose derivatives – that have high binding capacity to the mucosa for significant periods of time. Several studies include these polymers in vaginal formulations to increase their dwelling time at the site of action. If these formulations were to include a suitable mucoadhesive polymer or a polymer mixture, an optimum formulation could be developed for the controlled release of the drug in the area where HIV transmission occurs.[76]

Vaginal films

Quick-dissolving films are promising and attractive dosage forms that may provide an alternative platform for the vaginal delivery of microbicide drug candidates. These are thin strips of water-soluble polymers that dissolve when they are placed in the vaginal mucosa, releasing the active ingredient.[159] Vaginal films have advantages such as discreet use, no product leakage during use, not requiring an applicator for insertion and offering rapid drug release with minimal packaging and reduced waste.[169] Some of the possible disadvantages after administration are local irritation and influence on sexual intercourse. It should also be noted that their large-scale production today would be more complicated than the options described above, not because of the cost of the materials required, but because of the underdevelopment of the production resources. Women's preferences regarding the physicochemical characteristics of these films have been evaluated, and according to one study, they prefer smooth, thin, translucent, square films.[170]

The history of films as microbicides to prevent sexual acquisition of HIV began in a similar way to gels, since the first references found date from the 1990s, with vaginal films containing the spermicide nonoxynol-9. As with the gel, trials in women showed that continued use of nonoxynol-9 did not block HIV transmission and produced lesions in the vaginal tract that may increase the sexual transmission of STIs.[171,172]

Sometime after the failure of nonoxynol-9, films were developed containing other substances with potential activity

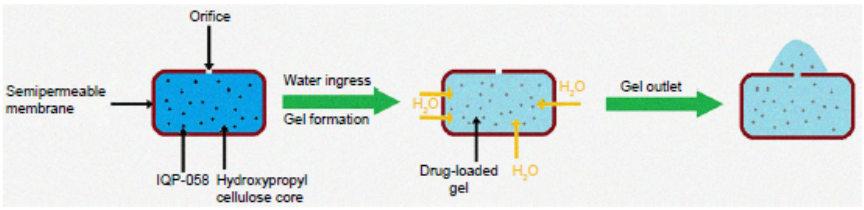

Figure 6 Mechanism of drug release from osmotic release tablets.

against HIV, such as sodium polystyrene sulfonate[173] and RC-101,[174] although none of them reached clinical trials.

Recent years have seen a growing interest in the development of microbicidal vaginal films, now produced with the incorporation of antiretroviral drugs. Films can be found incorporating NRTIs, such as a hydroxypropyl methylcellulose (HPMC) film containing abacavir, evaluated in vitro and in vivo in rabbits, and which has proven to be nonirritating to the vagina and capable of releasing the drug.[175,176] 4′-Ethynyl-2-fluoro-2′-deoxyadenosine is another NRTI that has been incorporated into films made with HPMC and polyvinyl alcohol (polyvinylpirrolidone [PVP]), both alone[177] and combined with 5-chloro-3-phenylsulfonylindole-2-carboxamide.[178] In vitro studies on these formulations corroborate their potential to inhibit HIV transmission.

Research has also been done on NNRTIs incorporated in vaginal films, of which DPV is unsurprisingly the most studied. These DPV-loaded films have been shown to prevent HIV-1 infection in vitro and ex vivo and have acceptable characteristics.[169] Recent Phase I clinical trials have demonstrated their safety and ability to release the drug and attain sufficient concentrations in the vaginal tissues to block HIV activity.[179] Further studies are thus awaited to evaluate the effectiveness of the formulation.[180]

There are also microbicidal films containing other NRTIs, such as a polyvinyl alcohol (PVA) film with the pyrimidinedione IQP-0528[181] and an HPMC and polyethylene glycol (PEG) 400 film with the antiretroviral UAM01398.[182]

The future of microbicides

Growing interest in nanosystems: nanomicrobicides

The high versatility of nanoparticles, which have transformed several fields of biomedical science, has led to intense research activity in this area in recent years.[183] These nanoparticles can be made using both inorganic materials and a wide variety of biodegradable and biocompatible polymers.

Thanks to their small size, they are able to internalize in cells and release the drug directly to the cytosol.[184] Their large surface area improves the dissolution and absorption of slightly soluble drugs, and also allows optimization of these nanoparticles according to their functionalization; they can bind to specific targets by multivalent conjugations and attach at the drug release site.[183–185] However, the complexity of the equipment required to obtain particles of this size would increase the cost of the formulation.

These nanosystems can either exhibit HIV inhibitory activity by themselves or serve as a vehicle for drug delivery.[183] Recent research has focused on the possibility of developing microbicides based on nanoparticles for HIV prevention. These nanoparticles consist of crosslinked polymer chains formed thanks to crosslinking agents, creating a structure within which to load the drug (Figure 7).

Their small size means that some of these particles can interact directly with HIV. In vitro viral adhesion has been observed with silver nanoparticles, and silver-coated PVP nanoparticles have demonstrated antiviral activity ex vivo at nontoxic concentrations.[186] This adhesion could be applicable to other noble metals such as gold.[187] The same researchers demonstrated the additive effect of the antiviral activity of silver nanoparticles when used in combination with monoclonal antibodies.[188]

Another option is to load nanoparticles with antiretroviral drugs, of which the most common are again TFV and DPV. For example, solid lipid nanoparticles of polylysine–heparin loaded with TFV have been shown to improve the cellular internalization of the microbicide and are not cytotoxic.[189] In the case of DPV, there are several references for nanoparticles based on polycaprolactone (PCL).[190,191] Other researches focused on developing nanoparticles of poly(D,L-lactic-co-glycolic acid) (PLGA) loaded with DPV, a formulation that has interesting technological and biologic characteristics for use in safe and effective vaginal microbicides. These nanoparticles are capable of releasing the drug for 24 hours at both pH 4.2 and 7.4, and their cytotoxicity is no higher than that of the free drug.[192] Another NNRTI evaluated for the prevention of sexual transmission of HIV is rilpivirine. PLGA nanoparticles have been developed with this drug and incorporated in a thermosensitive gel capable of offering significant protection against HIV-1 in mice.[106] However, most efforts to prevent HIV with rilpivirine do not focus on vaginal administration,

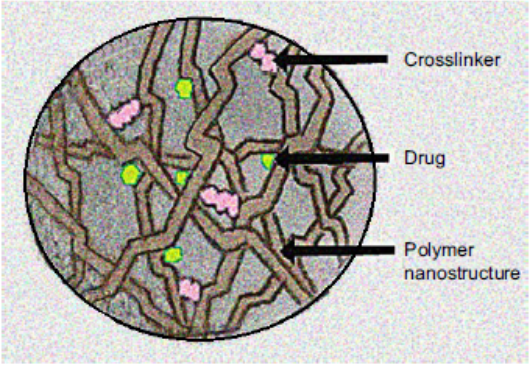

Figure 7 Structure of a drug-loaded polymer nanoparticle.

but on the intramuscular administration of long-acting rilpivirine.[104,105]

Once a nanogel that can retain the drug during loading and release it in a sustained way has been obtained, it is sometimes necessary to fit another set of parameters to affect the time of permanence of the nanoparticles and their concentration in the place they are required to exert their action.[193] The mucoadhesion can be changed by modifying the nanoparticle surface, for example, as in the case of chitosan nanoparticles loaded with TFV, which were coated with sodium triphosphate pentabasic to improve their mucoadhesion.[194] When these particles were made with a mixture of chitosan and thioglycolic acid, the mucoadhesion was even higher.[195]

Other modifications to the surface of the nanoparticles aim to improve their internalization in the vaginal mucosa. For example, PCL nanoparticles loaded with DPV have been developed and coated to assess how the surface charge affects their internalization in a simulated vaginal fluid medium incorporating mucin. The results of this study suggest that negatively charged particles are more suitable for the release of DPV into the vaginal mucosa, as they were able to pass through the medium much faster than positively charged particles.[196] Their ability to internalize in the cells was evaluated, and was observed to be greater than in the case of uncharged particles; there was also a correlation between the increase in intracellular drug release and antiviral activity.[190,191,197]

Finally, the nanoparticle surface can also be modified to increase its concentration at the site of action. Specific ligands are added to the surface and find their target at the drug's action site, allowing them to be retained and release the drug when they reach the target.[193] PLGA nanoparticles loaded with the antiretroviral drug saquinavir have been conjugated to the anti-CD4 antibody. The nanoparticles thus bind to the $CD4^+$ immune cells and the drug is specifically released inside them.[198]

Stimuli-sensitive materials are of significant interest in obtaining nanoparticles, since their physical and chemical nature can be modified in response to external stimuli such as pH or temperature. Nanoparticles of PLGA and methacrylic acid copolymer (Eudragit® S-100) have been loaded with TFV and are capable of releasing the drug in a pH-dependent manner in the presence of seminal fluid.[199] Another example of release in response to stimuli is the nanoparticles of hyaluronic acid loaded with TFV, which release the drug in the presence of semen due to the degradation of hyaluronic acid in the presence of the enzyme hyaluronidase.[200]

An alternative option is to include the nanoparticles in a stimuli-sensitive dosage form, such as temperature-sensitive gels that are liquid at room temperature, convenient to apply and gain consistency at body temperature, thus avoiding vaginal seepage after application and maintaining the nanoparticles in contact with the mucosa for longer. Formulations with PLGA nanoparticles loaded with TFV[201] or with rilpivirine[106] have been developed using these gels.

After manufacturing the nanoparticles, they must be properly formulated to develop vaginal microbicides that women can apply easily. The most common dosage form is as a gel, but the inclusion of nanoparticles in polymer films has recently been evaluated.[202] The advantages of nanoparticles combine with the benefits of the films described above; examples of this are PVA films incorporating nanoparticles loaded with small interfering RNA.[203] The incorporation of nanoparticles loaded with TFV or efavirenz into a film based on HPMC, PVA and glycerine was also evaluated,[204] and was found to produce sustained release of the drug for 24 hours and was found to be safe at in vivo trials.[205] Finally, it is worth mentioning another similar case in which PLGA nanoparticles were loaded with another antiretroviral, IQP-0528, and incorporated into a fast-dissolving film.[206]

For all these reasons, despite the current scarcity of microbicides based on nanosystems for the prevention of HIV, coming years will see a boom in research in this field, since nanoparticles provide a delivery strategy for targeted and controlled delivery of drugs to the vagina.[183,185]

Novel dosage forms: electrospun fibers

Although research based on more traditional pharmaceutical forms continues to be the main route for the development of vaginal microbicides, other less widely studied alternatives have been gaining importance in recent years and could offer a therapeutic alternative.

A recent new pharmaceutical form consists of drug-loaded polymer fibers known as electrospun fibers, which are produced by electrospinning, a technique that applies electrostatic forces to form polymeric fibers (Figure 8).[207] Polyglycolic acid, polylactic acid, PCL and PLGA are the most common polymers used in their manufacture.[208]

Unlike nanoparticles, which can internalize in the cells and release the drug, the fibers can only control the release of the drug through erosion and degradation of the polymer and diffusion of the incorporated drug. These materials offer an alternative to existing microbicides, as they can quickly – in <15 minutes – release the active ingredient in wet areas, especially if they contain a wetting agent as a carrier.[207,209]

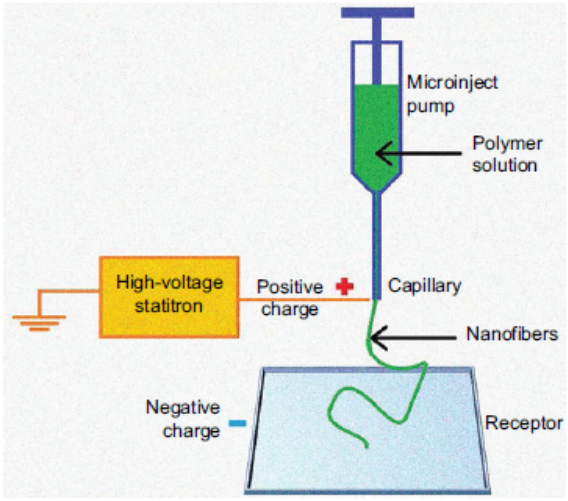

Figure 8 Diagram of the nanofiber manufacturing process by electrospinning.

The development of an effective microbicide in this formulation would be a useful tool for preventing HIV transmission, since its application prior to sexual relations would provide almost immediate protection. However, polymers that take longer to degrade in the vaginal medium can also be used in order to achieve sustained release of the drug, in some cases with over 7 days of sustained release.[207] As in the case of nanomicrobicides, a significant financial investment is required to purchase the equipment needed to obtain these formulations.

Some examples of vaginal microbicides formulated as electrospun fibers include TFV. PVA nanofibers loaded with this antiretroviral are capable of releasing 95% of the drug within 5 minutes in in vitro studies at a pH of both 4.3, equivalent to the pH of vaginal fluid, and 7.4, equivalent to the pH of seminal fluid.[210,211] Stimuli-sensitive, TFV-loaded nanofibers have also been developed based on thiolated hyaluronic acid, which release the drug only in the presence of semen, when the polymer degrades in the presence of the seminal hyaluronidase enzyme.[212]

Other nanofibers have incorporated antiretrovirals with a different mechanism of action, such as MVC, an entry inhibitor. A new study formulated electrospun solid dispersions of MVC with PVP or with poly(ethylene oxide). In the MVC in vitro release assays, 95% of the dose was released in 14 minutes when it was included in PVP fibers and in 18 minutes with the poly(ethylene oxide) fibers. When these electrospun fibers were manufactured with the addition of Tween 20 as a wetting agent, drug release was accelerated and at least 95% of the drug was released within 6 minutes.[209] As a variant of these MVC-loaded PVP fibers, more recently, PVP fibers in coaxially electrospun fibers were coated with ethyl cellulose, achieving sustained release of MVC for up to 120 hours.[213]

Finally, to highlight the rise of this new dosage form, it is worth noting that other antivirals are also being evaluated for this form of delivery, such as the recent development of electrospun nanofibers with griffithsin, which has shown in vitro efficacy against HIV-1 infection.[214]

This formulation could represent a major step forward in the development of vaginal microbicides, since it could improve their adherence regardless of their release rate. Rapidly dissolving electrospun fibers could offer immediate protection against the virus, and their use would be coitus dependent, so their administration would only be required prior to sexual intercourse. Fibers capable of delivering sustained release of the drug over several days would also offer a therapeutic advance in terms of adherence, since although the administration is coitus independent, daily applications would not be required.[207]

Genetically modified microorganisms: microbicidal probiotics

Although the potential of microbicidal probiotics as microbicides is still controversial, they are rapidly gaining acceptance. They work by promoting vaginal colonization by genetically modified microorganisms that can express molecules capable of inhibiting HIV. This strategy would require a high initial investment to genetically modify the microorganisms, but the possibility of these bacteria colonizing the vaginal medium would provide lasting protection that could offset such a substantial investment.

Currently under study are bacteria from the genera *Lactobacillus* and *Bifidobacterium*, common in the human vaginal microbiota, to determine whether these genetically modified microorganisms are stable, adhere and persist in the vaginal mucosa and produce the compound of interest in sufficient and sustained concentrations to inhibit the virus without damaging the normal balance of the vaginal microflora.[215,216]

A strain of *Lactobacillus jensenii* has been developed that is capable of colonizing mice vaginas and producing high levels of CV-N for long periods of time.[217] The same bacterial strain was studied in macaques, where it was demonstrated that its use is not only safe, but can also have a positive impact on the vaginal environment.[218] A subsequent

study showed that macaques colonized with this bacterium had a significant reduction in the transmission of a chimeric simian-HIV strain (SHIV-SF162P3).[219] The safety and toxicity of this strain have also been evaluated, as these are the most contested aspects when using probiotics as microbicides. In vivo tests on macaques have shown that their use is not only safe, but could also have a beneficial impact on the vaginal environment, with lower levels of inflammation in the macaques retaining this strain.[218] Another therapeutic alternative explored by the authors of these studies is the modification of this strain to express fragments of antibodies against HIV on its surface.[220]

Although there is still a need for more studies on the subject, this strategy undeniably represents an important step in the development of a lasting and inexpensive microbicide to block sexual transmission of HIV in women.

Broadening the spectrum of microbicides: combining drugs with different mechanisms of action

Following the successful combination of antiretroviral drugs for the treatment of HIV infection, strategies for microbicide development today involve designing dosage forms that combine drugs with different mechanisms of action which are released simultaneously in order to boost the effectiveness of the protection.

One widely studied combination is TFV, antiretroviral NRTI and DPV, an NNRTI, which have been used to develop polymeric films[15,221] and intravaginal polyurethane rings.[18] It was observed in ex vivo studies on the films that the coadministration of the two drugs caused a higher concentration of DPV in the tissue, whereas TFV was not affected. In the case of vaginal rings, both drugs were seen to be released together in vitro for 30 days.

Another well-studied combination is of DPV with the entry inhibitor MVC. Although films have also been developed that combine these two drugs,[15] this combination has been studied mainly for use in vaginal rings, since its objective is to improve the protection offered by successful DPV rings. After evaluating the combination of DPV with different amounts of MVC, a ring with 25 mg of DPV and 100 mg of MVC was selected for clinical trials.[222] Phase I trials demonstrated that the rings were safe and well tolerated,[223] but the plasma concentration of DPV was much higher when the ring contained both drugs than when it included only DPV. However, detectable concentrations of MVC were found only in cervicovaginal fluid, but not in plasma.[224]

Another option is the combination of TFV and MVC, used to develop intravaginal rings,[225] films[15] and even more novel formulations such as nanolipogels–nanoparticles whose core is a hydrogel wrapped in a lipid shell.[226]

Although TFV, DPV and MVC are the most commonly used drugs in antiretroviral combinations, alternative combinations of other active substances have also been evaluated, such as PLGA nanoparticles loaded with raltegravir and efavirenz, which are subsequently included in a thermosensitive ethyl cellulose gel.[227]

These studies demonstrate that combinations of antiretrovirals capable of inhibiting HIV transmission have potential as prevention strategies against sexual transmission of the virus.

There are a large number of vaginal microbicide formulations currently under investigation. Table 2 summarizes

Table 2 Vaginal formulations for the prevention of sexual transmission of HIV

Mechanism of action	Microbicide	Pharmaceutical form	Animal tests	Clinical trials	Current status	References
Surfactants	Nonoxynol-9	Gel Film	–	Not safe Not effective	Rejected	20, 24 171, 172
	Savvy gel®	Gel	–	Safe Not effective	Rejected	21, 23
Acidifier	BufferGel®	Gel	–	Safe Not effective	Rejected	32, 33
Polyanions	Carraguard®	Gel	–	Safe Not effective	Its use as a carrier is being assessed	25–28, 30
	PRO 2000®		Safe Effective	Safe Not effective	Rejected	32, 33, 35–37
	VivaGel®		Safe Effective	Not safe	Rejected	38–46

(*Continued*)

Table 2 (*Continued*)

Mechanism of action	Microbicide	Pharmaceutical form	Animal tests	Clinical trials	Current status	References
gp120-neutralizing monoclonal antibody	Vitamin B_{12}	Gel	Safe Dose-dependent effectiveness	–	Large amounts are required and it is very expensive to produce	50–52
	Cyanovirin-N	Gel	Safe Effective	–	Candidate for clinical trials It has been expressed and purified from transgenic plants	55–59
		Probiotics (genetically modified *Lactobacillus jensenii* strain)	Safe and positive for the vaginal environment Effective	–	In clinical trials	217–219
Entry inhibitors	Maraviroc	Gel (hydroxyethyl cellulose)	Safe Effective	–	Its period of effectiveness must be increased	69–71
		Gel (silicone)	Higher and sustained concentrations	–	Candidate for clinical trials	72, 73
		Intravaginal ring	Safe		Controlled release over 28 days	140
Viral enzyme inhibitors	Tenofovir/ tenofovir disoproxil fumarate	Gel	Safe Effective	Safe Effective	The first microbicide that demonstrated efficacy in women	80–89, 100
		Intravaginal ring	Safe Effective	Safe	In clinical trials It provides lasting protection in animals	16, 17, 132–138
		Nanoparticles (into a film)	Safe	–	Controlled release over 24 hours Further evaluation is needed	204, 205
	MIV-150	Gel	Effective	Safe	In clinical trials	107, 109–111, 113, 116
		Intravaginal ring	Effective	–	Candidate for clinical trials	141–144
	Dapivirine	Gel	Safe Effective	Safe	In clinical trials	119–123
		Intravaginal ring	Safe Effective	Safe Effective	Controlled release over 28 days Has demonstrated efficacy in women	151–156
		Film	Safe Effective	Safe	In clinical trials	169, 179

some of these and shows only those for which references to clinical trials or at least in vivo studies have been found. The different mechanisms of action and the various pharmaceutical forms give a clear picture of the benefits and drawbacks of each microbicide, and highlight the advances in these formulations. The ultimate goal is to achieve a vaginal microbicide that is lasting, safe, highly efficient and economical, in order to ensure protection of women against the acquisition of HIV.

Conclusion

On the basis of the above, it can be concluded that microbicides are a promising tool for the prevention of sexual transmission of HIV, although there is still a long way to go. The large number of microbicides tested in the last two decades have produced more failures than successes, but it is crucial to learn from the mistakes in ineffective formulations to develop an effective vaginal microbicide.

In the short term, microbicides based on reverse transcriptase inhibitors, such as TFV gel or DPV vaginal rings, are at a more advanced stage of development and have yielded the best results to date. It is imperative to continue the research into the potential of these drugs, so that other pharmaceutical forms can be developed to ensure women have multiple options for protecting themselves against infection with the virus.

In the long term, it is worth assessing other microbicides whose clinical application is currently far down the line, either because they are at an earlier stage of development – such as products created by nanotechnology, electrospun

solid dispersions or genetically modified microorganisms – or owing to the need to overcome certain barriers such as high cost, lack of adherence or difficulty in maintaining sustained concentrations.

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Authors' contributions

RRC and MDVO were responsible for the approach to this work, and all the authors participated in the design. FNP wrote the article, and MDVO and RRC conducted a critical review with important intellectual contributions. All three authors have approved the final version for publication.

MDVO, on behalf of the other signatories, guarantees the accuracy, transparency and truthfulness of the data and information contained in the study, and also that no relevant information has been omitted, and that all discrepancies between authors have been adequately resolved and described.

Disclosure

The authors report no conflicts of interest in this work.

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CAPÍTULO II

INFLUENCE OF CHITOSAN SWELLING BEHAVIOUR ON CONTROLLED RELEASE OF TENOFOVIR FROM MUCOADHESIVE VAGINAL SYSTEMS FOR PREVENTION OF SEXUAL TRANSMISSION OF HIV

marine drugs

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Article

Influence of Chitosan Swelling Behaviour on Controlled Release of Tenofovir from Mucoadhesive Vaginal Systems for Prevention of Sexual Transmission of HIV

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Abstract: The main challenges facing efforts to prevent the transmission of human immunodeficiency virus (HIV) are the lack of access to sexual education services and sexual violence against young women and girls. Vaginal formulations for the prevention of sexually transmitted infections are currently gaining importance in drug development. Vaginal mucoadhesive tablets can be developed by including natural polymers that have good binding capacity with mucosal tissues, such as chitosan or guar gum, semisynthetic polymers such as hydroxypropylmethyl cellulose, or synthetic polymers such as Eudragit® RS. This paper assesses the potential of chitosan for the development of sustained-release vaginal tablets of Tenofovir and compares it with different polymers. The parameters assessed were the permanence time of the bioadhesion—determined ex vivo using bovine vaginal mucosa as substrate—the drug release profiles from the formulation to the medium (simulated vaginal fluid), and swelling profiles in the same medium. Chitosan can be said to allow the manufacture of tablets that remain adhered to the vaginal mucosa and release the drug in a sustained way, with low toxicity and moderate swelling that ensures the comfort of the patient and may be useful for the prevention of sexual transmission of HIV.

Keywords: Human Immunodeficiency Virus; Acquired Immunodeficiency Syndrome; chitosan; mucoadhesive vaginal tablets; Tenofovir; controlled release; ex vivo bioadhesion; swelling behaviour; swelling witness microstructure

1. Introduction

Acquired Immunodeficiency Syndrome (AIDS) continues to be one of the main public health problems around the world, especially in countries with the fewest resources. It is estimated that 36.7 million people are currently living with HIV [1]. The latest available data indicate that significant progress has been made over the last decade [2–4], and yet HIV continues to highlight the world's inequalities. The main challenges in preventing HIV transmission are the lack of access to sexual education services, and sexual violence against young women and girls [5]. It is, therefore, necessary

to have methods such as microbicides that are controlled by women themselves in order to prevent transmission, so they no longer depend on men to prevent the acquisition of the virus.

Tenofovir (TFV) is a drug that acts by blocking reverse transcriptase activity in HIV infection. It is currently being investigated for its potential microbicidal effect against HIV [6,7]. TFV microbicide formulations have had proven antiviral efficacy in animal models and are currently in phase III clinical trials. Recent studies have demonstrated that TFV vaginal administration has no significant cytotoxicity in women and that TFV has no toxicity for vaginal mucosa at concentrations commonly used as a microbicide [8,9]. Numerous reports have assessed and confirmed the effectiveness of TFV vaginal formulations. A wide range of dosage forms containing this drug have been evaluated, including gels [10,11], films [12,13], and intravaginal rings [14–17].

Solid formulations have the advantage of high dose accuracy and long-term stability, as compared to semi-solid systems. The polymers used in these formulations must, therefore, be able to adhere to the vaginal mucosa and modulate drug release from the dosage form. The term "adhesion" describes the ability of certain macromolecules to adhere to the body's tissues; when this occurs in mucosa it is known as mucoadhesion. Although any material can adhere to the mucosa thanks to its viscous nature, there can be no real bioadhesion without an interrelation between some specific chemical groups in the polymers and biological tissues, or without establishing an interpenetration of chains. The dosage forms that bind to mucous membranes are described as mucoadhesive, as their purpose is to remain fixed at the point where the release and/or absorption of the drug occurs by prolonging its residence time [18,19].

All bioadhesive systems owe their properties to the inclusion of one or more types of polymeric molecules which, under appropriate conditions, establish interactions with the biological surface. One of these polymers is chitosan (CH), a natural, biocompatible, biodegradable, bioadhesive, and water-soluble polymer that degrades in acidic medium. It is obtained from the deacetylation of chitin, one of the most abundant polysaccharides in nature, as it is the structural element in the exoskeleton of crustaceans, such as crabs and shrimps. The amino and hydroxyl groups allow the adhesion to mucous through hydrogen bonds, and are protonated in an acid medium, which improves adhesion to negatively charged surfaces such as mucous. This polymer has been widely applied in the development of different pharmaceutical dosage forms for vaginal administration such as gels and tablets [20–23].

Possibly the most widely studied polymer for the development of such formulations is hydroxypropylmethyl cellulose (HPMC), a cellulose ether with methyl and hydroxypropyl groups used for the controlled release of drugs in hydrophilic matrix systems [24]. It is a FDA-approved polymer found in a wide range of applications, and was initially used in vaginal formulations as an excipient in the manufacture of films [25,26] and gels [27], although its use in vaginal administration tablets [20,28–30] has recently become more widespread. Another very similar polymer to HPMC and CH is guar gum (GG), which is also soluble in water, where it produces a viscous gel. GG is a biocompatible and biodegradable polysaccharide obtained from the seeds of *Cyamopsis tetragonoloba* used in the pharmaceutical industry as a binder or a disintegrant in tablets, and there are also several references to its use in the development of vaginal dosage forms. GG is sometimes present in bioadhesive vaginal gels, and has also been combined with HPMC to develop a bioadhesive vaginal tablet formulation [31,32]. All of the above-mentioned polymers are hydrophilic, and since the purpose of these formulations is their dissolution in the vaginal environment, it is also worth mentioning hydrophobic polymers such as Eudragit RS PO® (ERS). This is a copolymer of ethyl acrylate, methyl methacrylate, and a low content of methacrylic acid ester with quaternary ammonium groups. The ammonium groups are present as salts and render the polymers permeable. It is insoluble in aqueous medium and has low permeability and pH-independent swelling [33]. Its inclusion in pharmaceutical forms of vaginal administration to date is much scarcer than for the polymers described above, and it is mainly used in nanocapsules, microspheres, and microparticles [21,34,35].

With this background, the aim of this study is to assess the potential of chitosan to develop sustained release mucoadhesive tablets of TFV, where the drug release from these systems depends

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on the properties of each polymer. These properties are also analysed in other natural, semisynthetic, and synthetic polymers in order to assess the advantages offered by CH in the development of these formulations.

2. Results and Discussion

2.1. Swelling Tests

Figure 1 shows the swelling ratio (*SR*) profiles of the different batches studied. The maximum swelling ratio (SR_{max}) is included for each swelling curve. The curves in Figure 1 show that swelling and erosion processes are present in most cases. Hydrophilic polymers such as CH, HPMC, and GG swell when in contact with an aqueous medium as opposed to disintegrating. These batches increase in size due to the relaxation of the polymer chains. A temperature of 37 °C causes a decrease in the vitreous transition temperature, forming an area where the polymers change from a crystalline to a rubbery state (known as the gel layer) [36]. It is, thus, possible to distinguish a first stage for the GG and HPMC batches in which the swelling process takes precedence until the SR_{max} (96 h) is reached, followed by the erosion of the formulations. HPMC and GG are significant for having a high SR; this is higher in the case of GG, which also takes much longer to dissolve completely. GG is well known for its high water-absorbent capacity, which is the reason it is used in the development of superabsorbent hydrogels [37].

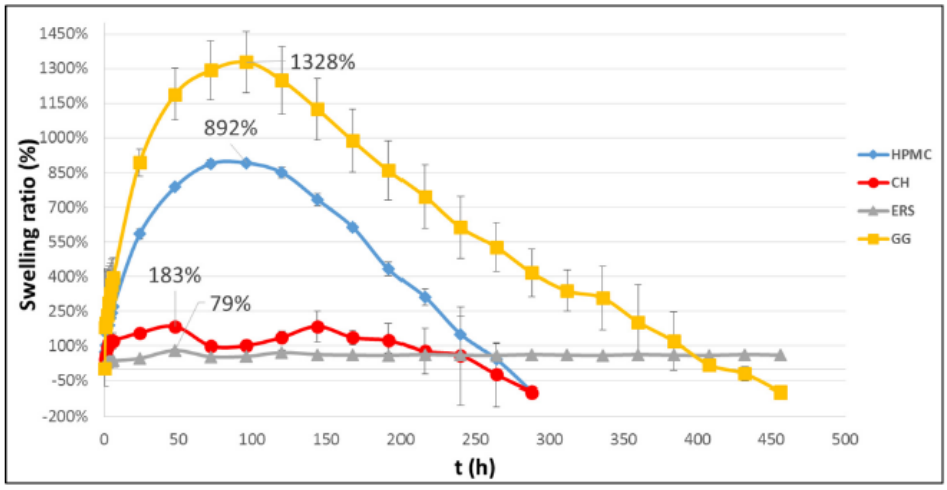

Figure 1. Swelling profile of each batch in simulated vaginal fluid (SVF). Data on the maximum swelling ratio (SR_{max}) are indicated.

In contrast, CH undergoes moderate but sustained swelling and acquires an aqueous volume of 183% of its weight, corroborating the results of Chen et al. [38]. This is because CH has an unusual swelling process, and after about 48 h the pressure from the gel causes the breakdown of its core (Figure 2). From the time when the fracture occurs, the core portion which the gel prevented from swelling is exposed to the aqueous medium and absorbs water, causing a new increase in SR values (Figure 1). Finally, ERS is not a water-soluble polymer and hardly absorbs water from the medium—although the porous matrix adsorbs a small amount—and, thus, remains undissolved throughout the test (19 days). This renders it inadequate, as once the drug has been released, the compact would need to be removed. In view of the results, the batch with CH would be the most comfortable, since it undergoes moderate swelling and complete erosion. This factor, the comfort of women, is crucial for the adherence to the use of the formulation. In this respect there is no problem since studies show that vaginal tablets are the solid dosage form preferred by women for intravaginal

administration [39]. In addition, the small size of the compacts developed (2.2–2.3 mm in height) makes them even more comfortable.

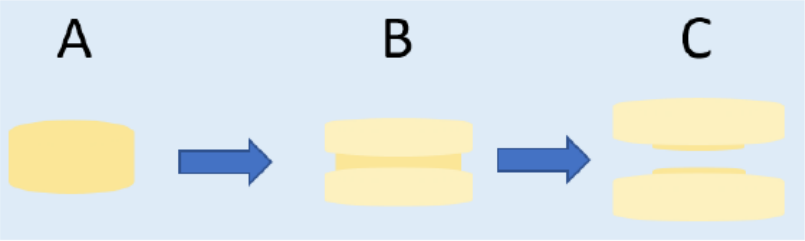

Figure 2. Chitosan compact swelling pattern. First the compact has a given shape (**A**), although the upper and lower layers swell in the presence of SFV, exerting pressure on the core (**B**) until finally this pressure causes the compact to break (**C**).

2.2. Release Study

A drug's release rate from a dosage form can be influenced by different phenomena, ranging from drug dissolution to water absorption, polymer swelling, and the dissolution and diffusion of the drug through the polymer network [40]. Figure 3 shows the TFV release profiles corresponding to the prepared batches. The release data shows that HPMC and CH are the polymers that best control the drug release from the compact. One interesting result is that in the first 48 h these formulations released lower drug amounts than those containing ERS or GG, owing to the characteristics of ERS and GG. ERS is an insoluble polymer with pH-independent swelling and low permeability that is unable to gel in aqueous medium and, thus, barely controls the release of the drug [33]. Although GG produces the gel layer with the highest *SR* (Figure 1), it has very little consistency and the drug diffuses rapidly through it, so it does not represent a delayed release mechanism [41]. However, when HPMC and CH compacts are introduced into simulated vaginal fluid (SVF) the outer layers form a strong consistency gel, as reported by other authors, which controls TFV release [20]. This result would ensure women were protected against the transmission of HIV for at least 3–4 days (90%–95% TFV released). The inhibitory concentration 50 (IC_{50}) of TFV has been found to be between 1.08–1.22 μM depending on the HIV strain used [42]. Thus, using these compacts IC_{50} is reached in a few minutes after administration.

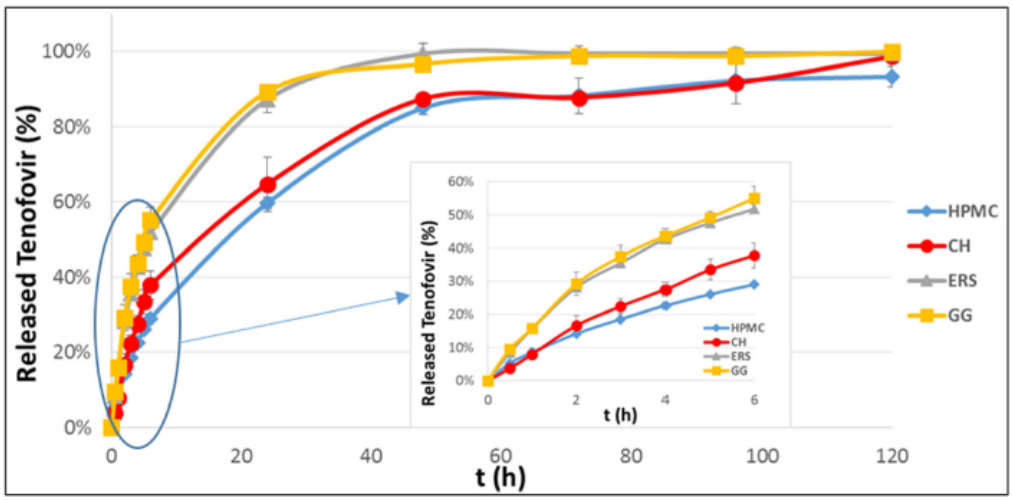

Figure 3. Tenofovir release profiles obtained from different batches in SVF.

In order to investigate the kinetics of TFV release from these formulations, mathematical model dependent methods (zero-order, first-order, Higuchi, Korsmeyer-Peppas, Hixson-Crowell, Hopfenberg

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and Weibull) were used to fit the experimental results. After analysing each batch, the models found to have the best fit to the curves in Figure 3 (high correlation coefficients R^2) were Korsmeyer-Peppas and Higuchi and Weibull [43,44].

According to Korsmeyer-Peppas, the drug release as a function of time follows Equation (1):

$$M_t/M_\infty = Kt^n \quad (1)$$

Which can also be expressed as Equation (2):

$$Ln(M_t/M_\infty) = Ln(K_{KP}) + nLn(t) \quad (2)$$

where M_t/M_∞ is the fraction of drug released at time t, K_{KP} is a constant incorporating the structural and geometric characteristics of the compact, and n is the release exponent [44]. In the case of cylindrical compacts, depending on the n value the drug release could follow a pure diffusion process ($n \leq 0.45$), an anomalous transport with simultaneous diffusion and structural modification of the polymer matrix ($0.45 < n < 0.89$), transport case II ($n = 0.89$) or transport Supercase II ($n > 0.89$). Both Case II and Supercase II involve the structural modification of the polymer matrix [43,45].

A good fit to the Higuchi model indicates that the drug diffuses through pores in the polymer matrix (this process is equivalent to Korsmeyer–Peppas for $n \leq 0.45$). The Higuchi equation for fitting the curves of Figure 3 is in this case Equation (3), where Q is the amount of drug released in time t and K_H is the Higuchi dissolution constant:

$$Q = K_H t^{1/2} \quad (3)$$

Finally, the curves in Figure 3 were also evaluated by the Weibull model, with the following mathematical equation:

$$M = M_0 \left[1 - e^{-\frac{(t-t_{lag})^b}{a}}\right] \quad (4)$$

where M is the dissolved drug, M_0 is the total amount of drug in the compact and t_{lag} is the lag time, a is a scale parameter that describes the dependence on time, and b describes the shape of the dissolution curve [43].

In our case, where there is no lag time (see curves in Figure 3), and because the drug release profiles have an exponential shape, b is equal to 1. If we take the constant $K_W = 1/a$, then Equation (4) can be summarised as Equation (5):

$$\ln\left(1 - \frac{M}{M_0}\right) = -K_W t \quad (5)$$

Figure 4 shows the corresponding fit of the experimental data to these drug release models, and Table 1 shows the n, K_{KP}, K_H, and K_W kinetic constants for these three models.

Table 1. TFV release kinetics from HPMC, CH, ERS, and GG batches.

Batch	Korsmeyer-Peppas			Higuchi		Weibull	
	K_{KP}	n	R^2	K_H	R^2	K_W	R^2
HPMC	0.088	0.63	0.9899	0.124	0.9980	0.036	0.9931
CH	0.077	0.92	0.9926	0.130	0.9815	0.040	0.9839
ERS	0.152	0.73	0.9887	0.148	0.9453	0.098	0.9859
GG	0.161	0.71	0.9929	0.145	0.9231	0.068	0.9756

According to the data in Table 1, the batches with HPMC and CH have a good fit with the Higuchi kinetic (Figure 4A). However, the Higuchi kinetic can only be applied when the swelling

and dissolution of the matrix are negligible [43], so it cannot be used to explain the drug release behaviour from these batches. All of the batches tested have a good fit to the Korsmeyer–Peppas kinetic (Figure 4B). The analysis of n values for different batches reveals that those prepared with HPMC, ERS and GG have similar values of close to 0.7, and that in the case of CH n is higher and close to 1. It can, therefore, be said that the TFV release from HPMC, ERS, and GG corresponds to an anomalous (non-Fickian) transport, while for CH—whose n value is over 0.89—it follows a Supercase II release, a mechanism that implies an extreme drug transport [45]. During polymer swelling the breakage of the compact occurs because the upper and lower layers of the compact swell to form a gel, causing a compressive stress on the core that prevents axial swelling. As the gel continues pressing on the core, the internal compact pressure increases until the core breaks (Figure 2) [46].

The n values for HPMC, ERS, and GG fall in the range 0.45–0.89, indicating that the drug release is governed by simultaneous structural modification and diffusion through the polymer matrix processes. In the HPMC and GG batches, the polymer swells at the same time as the drug diffuses through the gel formed. As has been shown in the swelling test, these two polymers form a long-lasting gel and the rearrangement of chains occurs slowly; the simultaneous diffusion is the process that causes the time-dependent anomalous effect [46].

ERS captures very little water, which rules out polymer swelling as a possible explanation. When the drug release from the ERS batch is fit to the Weibull model, the constant K_W is much higher than for the other polymers—including GG (Figure 4C)—although the differences between GG and ERS in the other models are insignificant (Figure 4A,B). The Kw values in the Weibull equation represent the drug release rate constant. HPMC and chitosan have similar K_W values that are much lower than the other two formulations, signalling the greater control over the release of TFV from the compacts made with these polymers. In contrast, GG and ERS have much higher K_W values, since the drug also diffuses from these compacts at a greater rate. The Weibull model is used to analyse the release profile of matrix-type drug delivery, and this is the mechanism of TFV release in ERS. This is because ERS is a permeable polymer that allows water into the compact, followed by the dissolution of the drug in the medium, and finally the diffusion of TFV through the polymer.

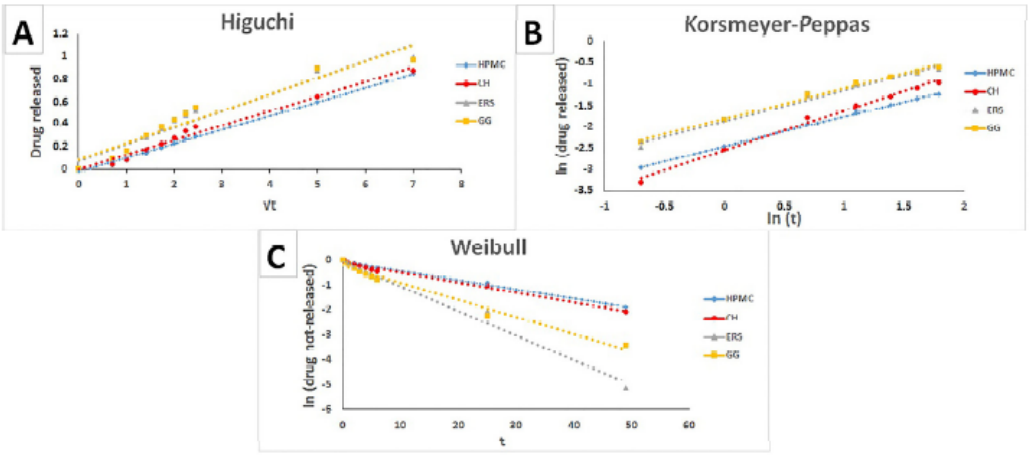

Figure 4. Fit of TFV release from batches HPMC, CH, ERS, and GG to the Higuchi (**A**) Korsmeyer-Peppas (**B**), and Weibull (**C**) models.

2.3. Microstructure of Witnesses. FE-SEM, and Hg Porosimetry

It is well known that water is removed during freeze-drying of a hydrated polymer system, and the space that was originally occupied by the solvent is transformed into pores, generating a porous structure similar to a sponge [47]. As can be seen from the micrographs in Figure 5, the witness microstructures vary considerably depending on the polymer type. Figure 5A, corresponding to the

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swelling witness of HPMC, has a channelled microstructure formed when water enters the polymer during swelling. These channels allow the compacts to maintain their shape while the drug diffuses slowly between them. This perfectly homogeneous microstructure is maintained because HPMC swelling occurs progressively; the outer layers become swollen but the core remains unswollen until the outer gel erodes and water reaches the core [48]. This is observed in our release studies, thus, water mobility plays a role in controlling drug release.

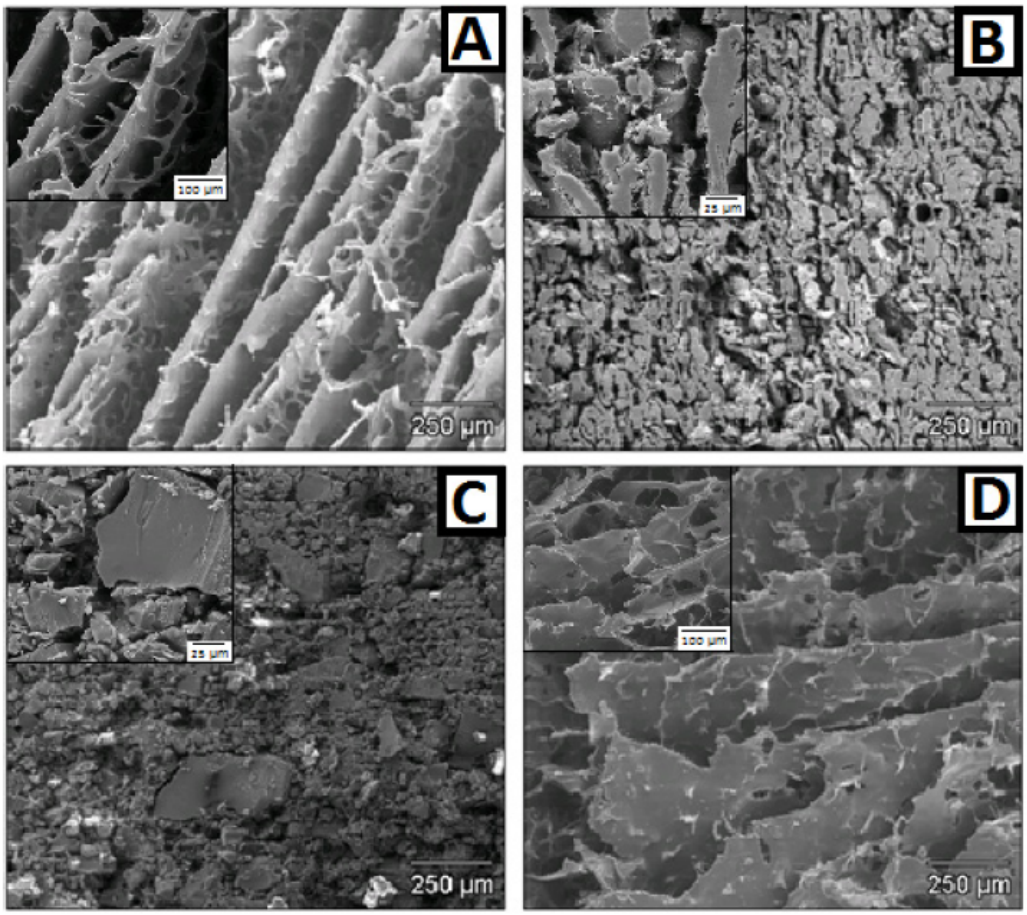

Figure 5. Electron microscopy micrographs of swollen witnesses of HPMC (**A**); chitosan (**B**); Eudragit® RS PO (**C**); and guar gum (**D**).

The micrograph of the CH witness (Figure 5B) shows a sponge-like microstructure with numerous pores in the polymer through which the SVF circulates, albeit with difficulty. This result explains the controlled release of TFV from CH in spite of its moderate swelling capacity. In contrast, no defined microstructure is observed for the ERS witness (Figure 5C); the formulation has a grainy microstructure with different-sized particles, but is unable to swell, which explains the failure of this formulation to control TFV delivery. Finally, the micrograph of the GG witness (Figure 5D) shows a perfectly microstructured formulation where the polymer is arranged in parallel sheets with the absorbed water between them. This microstructure explains why GG formulations swell the most and remain swollen the longest, as there is a high capacity for very effectively retaining water between these sheets. However, although the water cannot escape, the drug is able to diffuse through the polymer sheets, so their ability to retain the drug is minimal.

The above porous microstructures have been characterized by Hg porosimetry. Figure 6 shows the corresponding pore size distributions (PSD).

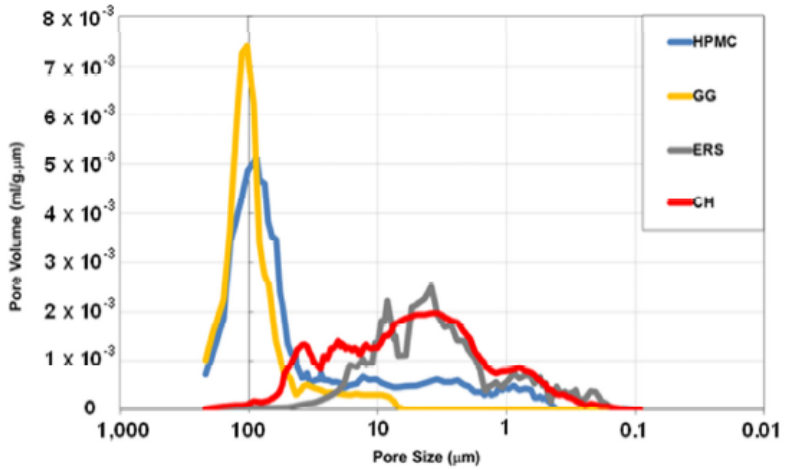

Figure 6. Pore distributions of HPMC, CH, ERS, and GG witnesses.

According to Figure 6, two types of PSD can be described: one has a narrow PSD where most of the pores are close to 100 µm, while the other has a wide PSD with pores of between 100 and 0.1 µm. Both PSD types are unambiguously associated to the swelling behaviours in Figure 1 and the FSEM micrographs in Figure 5. The HPMC and GG batches have high swelling characteristics with well-defined channelled microstructures and produce a narrow PSD with high pore sizes. In contrast, ERS and CH, with minimal swelling properties and grainy microstructures, produce a wide PSD with small pore sizes. There are two interesting observations: the first is that while the PSD of the GG batch also has a small number of pores with sizes between 50 and 10 mm, the PSD of HPMC also has small pores with sizes between 50 and 0.5 µm; and the other is that the PSD of CH has pores between 10 and 100 µm, while the PSD of ERS is below 50 µm. This points to the conclusion that as the compact has higher swelling properties, the corresponding PSD must have a high pore size.

Table 2 contains a summary of the results obtained from these PSD, and shows that mean pore size (*D*p) values are related to SR values, as mentioned earlier. Hence, the higher the swelling capacity, the higher the *D*p values. However, pore volumes (*V*p) are more closely related to the total number of pores present in the witness, so the lowest *V*p correspond to the ERS batch containing the smallest pore size. The CH witness has a higher *V*p value than ERS due to its larger pore size, as indicated by the *D*p. It is followed by GG, with a high *V*p value but lower than HPMC, although it has a higher *D*p. Finally, the highest *V*p corresponds to HPMC. HPMC's higher *V*p compared to GG is due to the small pores of between 50 and 0.5 µm in HPMC, but not in GG. In contrast, pore area (*S*p) values show the opposite pattern; namely the higher the *D*p, the lower the *S*p. This is because pore area increases as pore volume decreases. As may be expected, porosity (P) values are related to *D*p and *V*p values, as porosity increases with both pore size and pore volume. Finally, bulk density (ρ_B) values are related to *V*p and *P* values, as ρ_B corresponds to a sample where pores and material are measured as a whole. However, apparent density (ρ_A) corresponds to the sample with no pores over 0.1 µm, i.e., a dense sample, and these values are characteristic of the chemical sample composition.

Table 2. Pore volume (*V*p), pore area (*S*p), mean pore size (*D*p), bulk and apparent densities (ρ_B, ρ_A), and porosity (*P*) of HPMC, CH, ERS, and GG witnesses.

Witness	*V*p ($cm^3 \cdot g^{-1}$)	*S*p ($m^2 \cdot g^{-1}$)	*D*p (µm)	ρ_B ($cm^3 \cdot g^{-1}$)	ρ_A ($cm^3 \cdot g^{-1}$)	*P* (%)
HPMC	5.97	0.36	91.89	0.14	0.90	84
CH	1.74	0.43	28.74	0.38	1.19	67
ERS	0.35	3.59	9.16	0.77	1.06	27
GG	5.89	0.25	106.08	0.14	0.97	85

These results show that P, Vp, Dp, and ρ_B are related to the SR_{max} of the corresponding batches (Figure 1), but are not clearly related to the release profiles (Figure 3) or release kinetics (Table 1). Thus, the following relationship (with $R^2 = 0.992$) has been found between Dp and SR_{max}:

$$Dp = 35.4 \cdot Ln(SR_{max}) + 13.4 \quad (6)$$

This equation indicates that for a polymer with a very low swelling capacity the release of the TFV drug in aqueous medium causes pores of around 13 µm. In our case the ERS polymer presented pores with a mean size of 9 µm, close to the value obtained by this equation.

2.4. Evaluation of Mucoadhesion

An analysis of the mucoadhesion results (Figure 7) reveals that HPMC, ERS, and GG formulations remain attached to the mucosa for extended periods of time, even after all the TFV has been released. In contrast, the CH formulation shows a good initial adhesion to vaginal mucosa, and a residence time of about 48 h. This agrees with the results of other studies, highlighting the lower mucoadhesive ability of CH compared to cellulose derivatives [49]. This seems to be because the bonding to the mucosa by positively charged groups, as in the case of chitosan, is less durable than bonding through hydrogen bonds, which is typical of HPMC and GG.

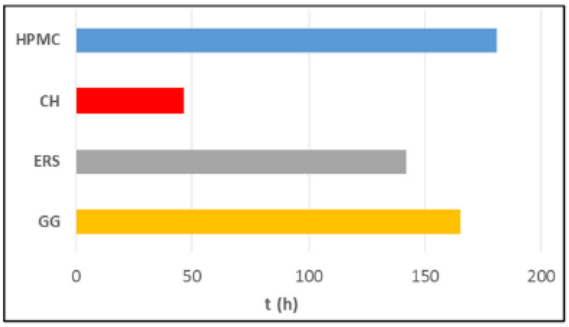

Figure 7. Mucoadhesion residence time of each batch in SVF.

The other three polymers show longer adhesion times to the mucosa of over 140 h in all cases. The formulation that remains attached for longest is HPMC, followed by GG and ERS. HPMC has been studied in depth, and this research corroborates its high mucoadhesive potential, caused by hydrogen bonding effects [50]. Although the mucoadhesive properties of GG have been poorly studied, another work shows its mucoadhesive strength is similar to HPMC [51]. Lastly, the most surprising results derive from the evaluation of the mucoadhesion of ERS, which in the previous literature is not classified as a mucoadhesive polymer. Our study shows that it has substantial mucoadhesive properties, and confirms a previous study comparing ERS with materials typically regarded in the literature as being good adhesives [52]. The adhesion to mucosa may be due to the presence of the quaternary ammonium group, which is protonated and may bind to negative charges in mucosa. Although these good mucoadhesion results highlight the high binding ability of the polymers, shorter times are required in this case—similar to the time for CH—since the TFV release occurs over a shorter period and there is no therapeutic justification for retaining the formulation adhered to the patient's vaginal mucosa after the drug has been completely released. In addition to discomfort, it could induce the rejection of the formulation.

2.5. Cell Toxicity

The biocompatibility of the formulations was evaluated through an in vitro cellular toxicity assay. All of the components of the different formulations were incubated at 37 °C in a 5% CO_2 atmosphere

for five days before the assay to ensure that any potential toxic component would be present in the suspension. MT-2, a lymphoblastoid cell line, and HEC-1A, a uterus-derived cell line, were seeded and treated with different dilutions of the suspensions. All of the components were tested at a maximum concentration of 1000 µg/mL in base-5 serial dilutions. Experiments were performed on MT-2 cells to evaluate toxicity on the immune cells present in vaginal or uterine mucosae, and also on the uterus epithelial cell line (HEC-1A) to assess any potential damage to the integrity of the mucosae. Cytotoxic concentration 50 (CC_{50}) were calculated when possible.

As shown in Table 3 and Figure 8, no toxicity was detected at the concentrations tested for any of the compounds. Interestingly, Tenofovir did not show cytotoxicity even at the highest tested concentration of 1000 µg/mL (around 3.3 mM).

Table 3. CC_{50} values of TFV, GG, CH, ERS, and HPMC obtained from the cytotoxicity assay in both MT-2 and HEC-1A cell lines. CC_{50}: cytotoxic concentration 50%.

Evaluated Substance	Cell Line	CC50
TFV	MT-2	>1000 µg/mL
	HEC-1A	>1000 µg/mL
GG	MT-2	>1000 µg/mL
	HEC-1A	>1000 µg/mL
CH	MT-2	>1000 µg/mL
	HEC-1A	>1000 µg/mL
ERS	MT-2	>1000 µg/mL
	HEC-1A	>1000 µg/mL
HPMC	MT-2	>1000 µg/mL
	HEC-1A	>1000 µg/mL

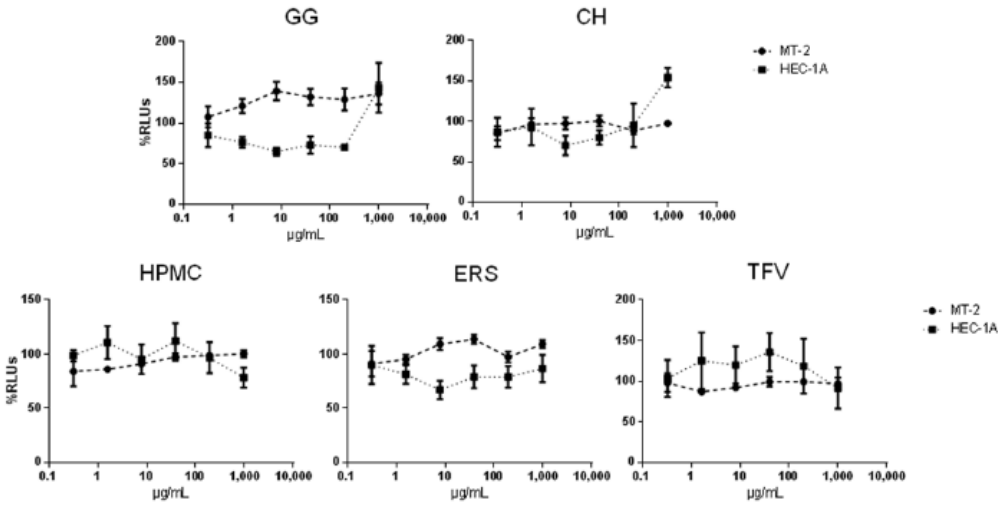

Figure 8. Cytotoxic evaluation of TFV, GG, CH, ERS, and HPMC measured in MT-2 cells and HEC-1A cells.

3. Experimental Section

3.1. Materials and Preparation of the Compact

Tenofovir (TFV, lot: FT104801401, MW: 287.21 g/mol) was supplied by Carbosynth Limited (Berkshire, UK). Chitosan, with 97% deacetylation and a viscosity of 92 mpa·s (CH, lot: 8826900003), was provided by Nessler (Madrid, Spain). The molecular weight, 10^5 g/mol, was estimated by viscometric measurements. Hydroxypropylmethylcellulose—Methocel® K 100 M (HPMC; lot:

DT352711, MW: 72 × 10^4 g/mol) was kindly supplied by Colorcon Ltd. (Kent, UK). Eudragit RS® (ERS; lot: G120238035, MW: 407.932 g/mol) was supplied by Evonik (Essen, Germany). Guar gum (GG; lot: SLBH5231V, MW: 22 × 10^4 g/mol) was acquired from Sigma-Aldrich (Saint Louis, MO, USA). Magnesium stearate PRS-CODEX (MgSt; lot: 85269 ALP) was acquired from Panreac (Barcelona, Spain). All other reagents used in this study were of analytical grade and used without further purification. Demineralized water was used in all cases.

Four batches of compacts were prepared from physical mixtures of the corresponding polymer (HPMC, CH, ERS, GG), TFV and MgSt. In all cases, each compact contained 290, 30, and 3 mg of polymer, TFV, and MgSt respectively.

In all cases the compacts were prepared with a press similar to the one used for preparing solid samples for analysis by IR spectroscopy. A stainless-steel disc was placed in the die, and the physical mixture of the components was placed on top. A second stainless steel disc was then placed on top. Five tons of constant pressure was applied using a punch for four minutes. Finally, the piston and the discs were removed, and the compact was stored in a desiccator until its subsequent evaluation. The manufactured compacts were cylindrical in shape and measured 13 mm in diameter and 2.2–2.3 mm in height.

3.2. Methods

3.2.1. Swelling Tests

The swelling pattern of the different batches in SVF were analysed using the method described by Ruiz-Caro et al. [53]. Each analysis was tested in triplicate. Swelling tests were carried out in a shaking water bath at 37 °C and 15 opm. In order to maintain the contact between the compact and the medium, and for more convenient handling of the samples during the test, each compact was previously fixed to a stainless steel disc, 3 cm in diameter, with a cyanoacrylate adhesive. At given time intervals (every hour during the first six hours and once a day for the remainder of the analysis), the discs were removed from the medium, placed on filter paper to eliminate the liquid excess and weighed. SR was calculated according to Equation (7):

$$SR = \left(\frac{C_s - C_d}{C_d} \right) \cdot 100 \quad (7)$$

where C_s and C_d correspond to the swollen and dry compact weights, respectively.

3.2.2. Release Study

The method described by Sánchez-Sánchez et al. [20] was used concurrently with the swelling test to assess the release behaviour of TFV in each batch. Each sample was inserted in a borosilicate glass bottle containing 80 mL of the SVF [54] and placed in a shaking water bath (Selecta® UNITRONIC320 OR, Barcelona, Spain) at 37 °C and 15 opm. Every day at given times, 5 mL samples were removed and filtered. The medium was replaced with the same volume of SVF at the same temperature. TFV release concentrations were quantified by UV spectroscopy at a wavelength of 260 nm in a Shimadzu® UV-1700 spectrophotometer (Kyoto, Japan). The test was performed in triplicate in each case. Studies have been conducted on the solubility of TFV in SFV at room temperature and the results are 4 mg/mL. Therefore, this ensures that the release study is performed under sink conditions and the release of drug is not conditioned by its solubility but by the release system.

The drug release experimental data were fitted to different model-dependent methods (zero order, first order, Higuchi, Korsmeyer-Peppas, Hixson-Crowell, Hopfenberg, and Weibull models) to investigate the kinetics of drug release from the various batches [43].

3.2.3. Swelling Witnesses

In order to characterize the microstructure acquired by the compacts when introduced in the SVF, swelling witnesses were prepared by determining the time each batch reached the maximum SR. This was done by attaching two compacts from each batch to the same stainless steel discs used for the swelling test. The discs were treated in the same way as in the swelling test. They were immersed in a beaker with SVF which was then placed in the shaking water bath (Selecta®UNITRONIC320 OR, Barcelona, Spain) (37 °C and 15 opm). The compacts were left under these conditions until they reached the maximum SR. Each compact was then extracted from the medium and lyophilized, and then stored in a desiccator until analysis. Witness microstructures were analysed by electron microscopy using a field emission scanning electron microscope (FE-SEM, Hitachi 4700, Tokyo, Japan) at an accelerating voltage of 15 V. Pore size distributions (PSD) were determined by mercury porosimetry using an Autopore II 9215 (Micromeritics Corp., Norcross, GA, USA). The corresponding pore volumes (Vp), pore areas (Sp), mean pore sizes (Dp), bulk and apparent densities (ρ_B, ρ_A), and porosities (P) of the samples were calculated from these PSD, assuming cylindrical pore shapes in all cases.

3.2.4. Assessment of Mucoadhesion

A new ex vivo mucoadhesion test was used to determine how long the compact remained adhered to the vaginal mucosa. A sample of freshly excised veal vaginal mucosa (obtained from a local slaughterhouse) was fixed with a cyanoacrylate adhesive to an 8.5 cm × 5 cm stainless steel plate (SSP). Each compact was then adhered to the mucosa, applying a given pressure (500 g for 30 s). The SSP was placed at an angle of 60° inside a beaker containing 150 mL of SVF, and this system was inserted in the shaking water bath (Selecta® UNITRONIC320 OR, Barcelona, Spain) at 37 °C and 15 opm. The bioadhesion time of each batch was assessed by visual observation of the samples. All batches were tested in duplicate.

3.2.5. Cytotoxicity Assessment

Two human cell lines were used: a lymphoblastic cell line, MT-2 [55] and a uterus/endometrium epithelial cell line, HEC-1-A (kindly provided by M. A. Muñoz, Hospital Gregorio Marañón, Madrid, Spain). Both cells were grown in RPMI 1640 medium supplemented with 10% (v/v) foetal bovine serum, 2 mM L-glutamine and 50 µg/mL streptomycin at 37 °C with a humidified atmosphere of 5% CO_2. HEC-1-A cells were detached by treatment with a trypsin 0.25% and EDTA 0.03% solution. Cell cultures were split twice a week.

Cell toxicity was measured by the CellTiter Glo viability assay (Promega). Briefly, GG, CH, ERS, HPMC, and TFV were suspended in water at a concentration of 10 mg/mL and left in culture (5% CO_2 and 37 °C) for five days [56]. Cells were then seeded in 96 microwell plates at a density of 1×10^5 cells per well in the case of MT-2, and 2×10^4 in the case of HEC-1-A, in complete RPMI medium, and treated with fresh medium containing different concentrations of suspensions (1000, 200, 40, 8, 1.6, and 0.32 µg/mL), or with the same concentration of vehicle (water). After 48 h of incubation, cell viability was measured following the manufacturer's instructions (CellTiter Glo viability assay), and RLUs were obtained in a luminometer. Data were normalized using RLUs obtained from cells treated with vehicle (100%) as a reference. Values of CC_{50} were calculated using GraphPad Prism Software (non-linear regression, log inhibitor versus response).

4. Conclusions

Vaginal compacts can be a very useful tool for the prevention of HIV transmission from men to women. Adherence to the use of microbicides has been one of the main drawbacks in demonstrating the efficacy of the formulations studied to date. In contrast, these compacts would decrease the frequency of administration by achieving sustained release of the drug over several days, resulting in a greater adherence to the treatment.

From the results obtained it can be concluded that there are two polymers (CH and HPMC) with the potential to achieve sustained and complete release of TFV from vaginal mucoadhesive compacts. However, the good bioadhesive properties of CH, which allow the formulation to remain attached to the vaginal mucosa only until all of the drug has been released, its moderate SR, which ensures more comfort for the patient than the other polymers tested, and its low cytotoxicity warrants that CH compacts containing TFV have proven to be a suitable formulation for the prevention of sexual transmission of HIV.

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CAPÍTULO III

OPTIMIZATION OF TENOFOVIR RELEASE FROM MUCOADHESIVE VAGINAL TABLETS BY POLYMER COMBINATION TO PREVENT SEXUAL TRANSMISSION OF HIV

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Research Paper

Optimization of tenofovir release from mucoadhesive vaginal tablets by polymer combination to prevent sexual transmission of HIV

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ABSTRACT

The use of sustained-release mucoadhesive vaginal tablets of antiretroviral drugs as microbicidal formulations can be an effective strategy for reducing the sexual transmission of HIV from men to women, which is a main problem particularly in low- and middle-income countries. Different polymers (hydroxypropylmethyl cellulose (HPMC), chitosan, guar gum and Eudragit® RS) have proven some good features for this purpose. At this work, these polymers have been combined in pairs in different proportions to enhance the advantages offered by each one individually. The *in vitro* release of tenofovir from the matrices, *ex vivo* mucoadhesive capacity (evaluated on vaginal mucosa) and the degree of swelling in simulated vaginal fluid have been assessed. A multimodal pore size distribution is observed in porosimetry studies -carried out with swelling witnesses-, due to the contribution of polymers with different swelling behaviour to the pore formation, and it is corroborated by scanning electron microscopy. X-ray diffraction technique confirms the changes in crystallinity of the formulation after swelling. We can report that the combination of HPMC and chitosan in the same formulation may be useful for the prevention of sexual transmission of HIV, since tablets can be obtained that remain adhered to the vaginal mucosa for 96 h, so the drug is released in a sustained manner for 72 h. When the formulation contains more chitosan than HPMC the swelling is moderate, making it more comfortable for women to apply.

1. Introduction

Acquired immunodeficiency syndrome (AIDS) is a chronic infectious disease caused by the human immunodeficiency virus (HIV). The number of people living with HIV continues to grow, largely because more people in the world have access to antiretroviral treatment and are thus able to live longer and healthier lives. The number of new infections and the mortality rate continues to decrease worldwide. Nonetheless, it is estimated that only about half the people living with HIV know their HIV status (UNAIDS, 2015; World Health Organization, 2007).

Despite the progress made in the last decade, AIDS is not yet a thing of the past. Every year, about one million women and girls become HIV infected. It is therefore necessary to focus efforts on a method for preventing HIV transmission that will significantly reduce the number of new infections. Worldwide, young women are twice as likely to acquire HIV as their male counterparts, so women from low- and middle-income countries should be specifically targeted, since they account for the vast majority of new cases. Approximately 75% of young women aged 15–19 report that they do not have a final say in decisions about their own health. Three out of four new infections in sub-Saharan 15–19-year-olds are among girls, and 80% of young women with HIV live in sub-Saharan Africa (UNAIDS, 2014, 2017). The development of a vaginal microbicide capable of significantly decreasing the sexual acquisition of the virus would provide women with a method to protect themselves, thereby reducing new cases of AIDS.

Tenofovir (TFV) is an antiretroviral drug, specifically a nucleoside reverse transcriptase inhibitor (NRTI), used for the treatment of HIV

Abbreviations: ACDP, anhydrous calcium hydrogen phosphate; AIDS, acquired immunodeficiency syndrome; $AUC_{0\rightarrow t_\infty}$, area under the curve between time 0 and complete erosion; CH, chitosan; Dp, mean pore sizes; ERS, Eudragit® RS; FE-SEM, field-emission scanning electron microscopy; GG, guar gum; HIV, human immunodeficiency virus; HPMC, hydroxypropylmethyl cellulose; MgSt, magnesium stearate; NRTI, nucleoside reverse transcriptase inhibitor; P, porosity; PSD, pore size distributions; PVP, polyvinylpyrrolidone; Sp, pore area; SR, swelling ratio; SR_{max}, maximum swelling ratio; SVF, simulated vaginal fluid; t_{SRmax}, time when the SR_{max} is reached; TFV, tenofovir; t_∞, time until complete erosion of the system; Vp, pore volume; ρA, apparent density

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infection. Its administration has been approved for the treatment of HIV-1-infected patients aged two and over (EMA, 2009). TFV antiretroviral activity has been confirmed and its oral use approved, and efficacy and safety studies have been conducted for the development of vaginal pharmaceutical forms with this active substance (Abdool Karim et al., 2010; Mayer et al., 2006). This is one of the most widely studied drugs for the development of vaginal microbicides, mainly since the CAPRISA 004 study, which demonstrated the efficacy in clinical trials of a 1% TFV gel in reducing sexual transmission of HIV (Abdool Karim et al., 2010; McConville, Boyd, & Major, 2014).

Adhesion to the vaginal mucosa is a crucial fact for the success of topical pre-exposure prophylaxis. TFV gels were evaluated to guarantee their mucoadhesion, using porcine vaginal tissue for *ex vivo* tests (Zidan & Habib, 2014). Other formulations developed later, such as TFV microparticles, were prepared with mucoadhesive polymers, such as chitosan (CH), for a longer dwelling of the formulation at the site of action (Khan & Thakur, 2014; Meng, Sturgis, & Youan, 2011). In another study were developed TFV loaded nanoparticles, which were subsequently included in mucoadhesive films of hydroxypropylmethyl cellulose (HPMC) and polyvinyl alcohol to achieve the adhesion of the formulation (Machado et al., 2016). Therefore, it is clear that the ability of the formulation to be adhered to vaginal tissue must be sought in the development of microbicidal products, and this is possible thanks to the use of polymers with proven mucoadhesive capacity as excipients.

Solid formulations have higher dose accuracy and longer stability compared to semi-solid systems. We found a few examples of TFV vaginal tablets for HIV prevention (Clark, Peet, Davis, Doncel, & Friend, 2014; McConville, Friend, Clark, & Malcolm, 2013; Pereira et al., 2014). These tablets were shown to be safe (Pereira et al., 2014), and TFV concentrations in the vaginal environment with this formulation were similar to those achieved with the 1% TFV gel, which proved effective in clinical trials (Clark et al., 2014). These formulations were designed as fast-dissolving vaginal tablets which, like the gel, would require a daily application (McConville et al., 2013).

Research is under way into the possibility of manufacturing vaginal controlled-release tablets of TFV formulated with mucoadhesive polymers that have high binding capacity to mucosa for significant periods of time. HPMC, CH, Eudragit® RS (ERS) and guar gum (GG) are considered mucoadhesive polymers since they have shown an ability to bind to the vaginal mucosa (Abruzzo, Cerchiara, Bigucci, Gallucci, & Luppi, 2015; Gupta, Natasha, Getyala, & Bhat, 2013; Hani, Shivakumar, Osmani, Srivastava, & Kumar Varma, 2016; Odeniyi, Khan, & Peh, 2015; Panda, Subhadarsini, & Mallick, 2016; Shitrit & Bianco-Peled, 2017), although remaining attached for different lengths of time (Notario-Pérez et al., 2017). The mucoadhesion of these polymers is a key feature, since it must be ensured that the tablets remain in the vagina as they take several days to release the drug completely. In these tablets the drug is uniformly distributed within the polymers, and drug release depends on the type, amount, particle size and solubility of the polymers, the solubility and particle size of the drug, and the geometry of the matrix (Sánchez, Damas, Domínguez, & Cerezo, 2010). The polymers included in these formulations must be able to modulate drug release from the dosage form and adhere to the vaginal mucosa.

Hydrophilic polymers capable of gelling in aqueous medium are an excellent option for developing these sustained-release tablets (Pygall, Kujawinski, Timmins, & Melia, 2017). GG is characterized by a rapid swelling of the formulation, resulting in a low-viscosity gel. HPMC undergoes swelling by layers; first, the outer layers of the tablet gel, maintaining a dry core on their interior which does not swell until the gelled layers have eroded. In contrast, CH has a pH-dependent swelling. Axial swelling of the upper and lower layers of the tablet occurs in vaginal fluid (pH 4.2) (Owen & Katz, 1999), but the intermediate layer is unable to gel due to the pressure exerted, which eventually leads to the breakdown of the tablet. Lastly, ERS is a hydrophobic polymer incapable of capturing water (Notario-Pérez et al., 2017). However, the uptake of water that allows the drug release to be modulated may also be a drawback for its clinical application, since excessive swelling would be uncomfortable for the user and could result in the patient ceasing to use the formulation.

The biocompatibility of the above-mentioned polymers (HPMC, CH, ERS and GG) and the drug (TFV) was previously corroborated through cytotoxicity assays in two human cell lines: a lymphoblastic cell line (MT-2) and a uterus/endometrium epithelial cell line (HEC-1-A) (Notario-Pérez et al., 2017).

In this scenario, the aim of this study is to combine polymers with different swelling and mucoadhesive characteristics to improve their individual features. The formulations must achieve the longest possible sustained release profiles, where the drug release is due to the contribution of different polymer properties, bioadhesion to the mucosa for the time required to completely release the drug, and comfortable swelling profiles for its clinical application.

Authors hypotheses is that the combination of polymers with high swelling ability (HPMC and GG) with polymers that capture little or nothing water (CH and ERS) in the same formulation can allow the development of a sustained delivery vaginal system of TFV (drug release at least for 72 h), able to remain attached to the vaginal mucosa during the 72 h of release and with a moderate uptake of vaginal fluid (capture of less than 500% of its weight in water) that would make it comfortable for women. Polymers combinations have shown in previous study their ability to control the release of water-soluble drugs (Cong et al., 2017; Verma, Dubey, Verma, & Nayak, 2017). Thus, its potential for this application deserves to be tested.

2. Materials and methods

2.1. Materials

Chitosan (CH, lot: 8826900003), with 97% deacetylation and a viscosity of 92 mPa s, was provided by Nessler (Madrid, Spain). The molecular weight, 10^5 g/mol, was estimated by viscometric measurements. Guar gum (GG; lot: SLBH5231V) was acquired from Sigma-Aldrich (Saint Louis, USA). The molecular weight of guar gum is not determined by the supplier but is estimated according to the literature -indicated by the suppliers themselves – at $22{\cdot}10^4$ g/mol. Hydroxypropyl methylcellulose – Methocel® K 100 M (HPMC; lot: DT352711) was kindly provided by Colorcon Ltd. (Kent, England). HPMC is only characterized by the supplier in terms of its viscosity (in 2% H_2O solution at 20 °C), which in this case is 121,894 mPa s. Based on this, the molecular weight is estimated at $72{\cdot}10^4$ g/mol. Eudragit RS® (ERS; lot: G120238035), with a molecular weight of 407.932 g/mol, was supplied by Evonik (Essen, Germany). Anhydrous calcium hydrogen phosphate – Emprove® (ACDP; lot: K93487944416) was supplied by Merck (Darmstadt, Germany). Polyvinylpyrrolidone – Kollidon® 30 (PVP; lot: 98-0820) was purchased from BASF Aktiengesellschaft (Ludwigshafen, Germany). Tenofovir (TFV, lot: FT104801401), with a molecular weight of 287.21 g/mol, was supplied by Carbosynth Limited (Berkshire, United Kingdom). Magnesium stearate PRS-CODEX (MgSt; lot: 85269 ALP) was acquired from Panreac (Barcelona, Spain). All other reagents used in this study were of analytical grade and used without further purification. Demineralized water was used in all cases.

2.2. Preparation of tablets

Samples of tablets from compacted granules were prepared combining two different polymers in three different proportions, seeking to supplement the properties of one polymer with the others. Table 1 shows the different batches produced. Each batch was prepared by mixing the polymers, ACDP and TFV. The mixture was then wetted with a solution of PVP in ethanol to produce a mass, which was granulated by passing through a 0.5 mm mesh. The granulate obtained was dried at

Table 1
Composition (mg/unit) of tablets.

Samples	HPMC	CH	ERS	GG	ACDP	PVP	TFV	MgSt
HPMC/CH 1	145	145			45	27	30	3
HPMC/CH 2	190	100			45	27	30	3
HPMC/CH 3	100	190			45	27	30	3
HPMC/ERS 1	145		145		45	27	30	3
HPMC/ERS 2	190		100		45	27	30	3
HPMC/ERS 3	100		190		45	27	30	3
GG/CH 1		145		145	45	27	30	3
GG/CH 2		100		190	45	27	30	3
GG/CH 3		190		100	45	27	30	3
GG/ERS 1			145	145	45	27	30	3
GG/ERS 2			100	190	45	27	30	3
GG/ERS 3			190	100	45	27	30	3

room temperature for 24 h. Before compression, 0.75% (w/w) MgSt was added to the total dry granulate.

The tablets were manufactured using a press similar to that used to prepare the solid samples analysed by IR spectroscopy. A constant pressure of 5 t was exerted for four minutes. The tablets were cylindrical in shape, and had a diameter of 13 mm and a height of 2.3–2.5 mm.

2.3. Assessment of the tablets

2.3.1. Release study

To evaluate the release and dissolution behaviour of TFV from the samples, each tablet was immersed in 80 mL of simulated vaginal fluid (SVF) (Owen & Katz, 1999) in a borosilicate glass bottle and placed in a shaking water bath (Selecta® UNITRONIC320 OR, Barcelona, Spain) at 37 °C and 15 opm. TFV concentrations in the SVF were quantified by UV spectroscopy at a wavelength of 260 nm in a Shimadzu® UV-1700 spectrophotometer (Kyoto, Japan). The assay was performed in triplicate for each sample.

In order to compare the effect of the polymers on TFV release, a model independent index described by Moore and Flanner was used (Moore & Flanner, 1996). This similarity factor (f_2) was calculated according to Eq. (1).

$$f_2 = 50 \times \log\left\{\left[1 + \left(\frac{1}{n}\right)\sum_{j=1}^{n} W_j |R_j - T_j|^2\right]^{-0.5} \times 100\right\} \quad (1)$$

Being n the number of samples for each dissolution test, R_j and T_j the drug release percentage at each time for the reference and test product respectively and W_j a weigh factor ($W_j = 1$ in this work). A value for $f_2 > 65$ indicates similarity between profiles higher than 95%, while $f_2 < 65$ denotes non-similar profiles (Mamani, Ruiz-Caro, & Veiga, 2012).

Experimental data on TFV release were also fitted to different model-dependent methods (zero order, first order, Higuchi, Korsmeyer-Peppas, Hixson-Crowell, Hopfenberg and Weibull models) to investigate the kinetics of drug release from the different samples (Dash, Murthy, Nath, & Chowdhury, 2010).

A fit to the Higuchi model indicates that the drug release occurs through a diffusion process based on Fick's law (Jain & Jain, 2016), a very common mechanism in matrix tablets. The Higuchi equation for fitting the experimental data is in this case Eq. (2), where Q is the amount of drug released at time t and K_H the Higuchi dissolution constant (Costa & Sousa Lobo, 2001; Jain & Jain,2016).

$$Q = K_H t^{1/2} \quad (2)$$

The Weibull model is commonly used to compare the drug release profiles from matrix-type systems (Dash et al., 2010). It is expressed as Eq. (3):

$$M = M_0\left[1 - e^{-\frac{(t-t_{lag})^b}{a}}\right] \quad (3)$$

where M is the drug released at time t, M_0 is the total amount of drug included in the formulation, t_{lag} is the lag time, a is a scale parameter that describes dependence on time and b takes different values as a function of the shape of the dissolution curves.

Eq. (4) has been used to fit the TFV release curves to the Weibull model, since t_{lag} always has a value of zero for our tablets, and the curve has an exponential shape ($b = 1$) (Costa & Sousa Lobo, 2001). K_w represents the Weibull constant, corresponding to $1/a$.

$$\ln\left(1 - \frac{M}{M_0}\right) = -K_W t \quad (4)$$

However, the mathematical model-dependent method that gives us more information about the mechanism of drug release from the tablets is the Korsmeyer-Peppas model. In this method, the drug release as a function of time can be expressed as Eq. (5):

$$M_t/M_\infty = K_{KP} t^n \quad (5)$$

where M_t/M_∞ represents the fraction of drug released at time t, K_{KP} is a constant that incorporates the geometric and structural characteristics of the tablet, and n is the release exponent (Costa & Sousa Lobo, 2001; Mamani et al., 2012). This model defines the mechanism of drug release according to the value of n.

2.3.2. Swelling tests

The influence of the types of polymer and their ratio on drug release is related to the swelling behaviour. The same shaking water described before, at 37 °C and at a speed of 15 opm, was used for the swelling tests. Each sample was tested in triplicate in SVF. Each tablet was fixed to a stainless-steel disc, 3 cm in diameter, with a cyanoacrylate adhesive (Loctite®, Henkel, Austria). This ensures constant contact is maintained between the tablet and the medium, and provides better handling of the samples during the test. The samples were extracted at given times, liquid excess was removed and they were weighed on a precision balance in order to calculate the swelling ratio (SR) according to Eq. (6):

$$SR = \left(\frac{C_s - C_d}{C_d}\right) \times 100 \quad (6)$$

where C_s and C_d correspond to the swollen and dry tablet weights respectively.

2.3.3. Swelling witnesses

To prepare swelling witnesses the tablets were fixed to a stainless-steel disc and immersed in SVF inside a beaker, which was then placed in the shaking water bath (37 °C and 15 opm). The tablets were maintained under these conditions until the maximum SR was reached (previously quantified in the swelling test), when they were extracted from the medium and lyophilized (Lio-Labor®; Telstar, Barcelona, Spain) to remove water and obtain a rigid porous structure called the swelling witness. Witness micrographs were taken by electron microscopy using a field emission scanning electron microscope (FE-SEM, Hitachi 4700) at an accelerating voltage of 15 V. Mercury porosimetry was performed with an Autopore II 9215 (Micromeritics Corp.) to determine pore size distributions (PSD). The corresponding pore volumes (Vp), pore areas (Sp), mean pore sizes (Dp), apparent densities (ρA) and porosities (P) of the swelling witnesses were calculated from these PSD, assuming cylindrical pore shapes.

2.3.4. X-ray diffraction

The X-ray diffraction patterns of raw materials, as well as tablets and swelling witnesses systems with polymers in equal ratios, were recorded by using an automated Philips® X'Pert-MPD X-ray

diffractometer with Bragg–Brentano geometry. Samples were irradiated with monochromatized Cu-Kα radiation (λ = 1.5406 Å) at 45 kV, 40 mA and a time per step of 2 s and analyzed between 2Θ angles of 5° and 50°.

2.3.5. Evaluation of mucoadhesion

A sample of freshly excised veal vaginal mucosa obtained from a local slaughterhouse was used as substrate on which to evaluate the residence time of the tablets adhered to the mucosa. The mucosa was fixed to a stainless-steel plate measuring 8.5 × 5 cm, and a tablet was placed on top, applying an identical quantified pressure (500 g for 30 s) for all samples. The plate was immersed in 150 mL of SVF inside a beaker at an angle of 60° and placed in the shaking water bath (37 °C, 15 opm). All samples were tested in duplicate, and bioadhesion time was defined as the period from the point the samples were introduced in the SVF until the tablet was observed to become detached from the mucosa.

3. Results and discussion

3.1. Release study

All formulations developed show sustained release of TFV during 48–72 h. However, differences can be observed between the drug release profiles of each formulation (Fig. 1). The mechanisms controlling the release of TFV from the tablets are the gelling rate of the hydrophilic polymers, the rate of drug diffusion, and the erosion rate of the gel.

As can be seen in Fig. 1A, tablets combining HPMC and CH have very similar TFV delivery profiles in all cases, achieving tablets that allow controlled release of the active ingredient regardless of the proportions of HPMC and CH in the sample. These results were somewhat predictable, since HPMC and CH individually had a similar drug release behaviour (Notario-Pérez et al., 2017). However, after their evaluation it can be verified that regardless of the proportion in which they are combined, the tablets maintain the excellent control over the drug release shown by both polymers, confirming the potential of the HPMC/CH combination for sustained drug release, as described by other authors (Cong et al., 2017; Verma et al., 2017).

When comparing tablets with HPMC and ERS (Fig. 1A), no interaction can be observed between the polymers, and the integrity of the formulation is maintained by the presence of HPMC, which forms a very thick gel (Gohel, Patel, & Bariya, 2003). In all cases an intermediate release profile is achieved between the profiles for the polymers separately (Notario-Pérez et al., 2017), obtaining greater drug release control as the proportion of HPMC in the formulation increases. In any case, since the control of the release is not higher than for HPMC as a single polymer, the HPMC/ERS combination offers no improvement in control over TFV release, as ERS is a hydrophobic polymer that is unable to swell (Evonik Industries, 2015; Notario-Pérez et al., 2017), and it has a lower ability to control drug release than HPMC.

Fig. 1B shows samples containing GG and CH. Although, of the two polymers combined in these formulations, CH has better control over release, improved control is achieved when they are combined in equal proportions, obtaining a release profile similar to the results for CH alone (Notario-Pérez et al., 2017). This is due to the fact that when CH is present in a greater proportion in the GG/CH combination, the gel formed during the dissolution study is unable to maintain its structure, and collapses in the first hours. This is shown in Fig. 1B, where is clearly distinguishable that sample GG/CH 3 causes an abruptly release of the drug, with a better releasing TFV performance. In contrast, the combination of both polymers in equal proportions, or with a greater proportion of GG, forms a gel that maintains its structure. These combinations were studied by Syed et al., who evaluated them at a pH of 1.2 and 7.2, and by Elkhodairy et al. at pH 1.2 and 6.8, observing a decrease in the drug release ratio compared to the formulation with GG alone (Elkhodairy, Elsaghir, & Al-Subayiel, 2014; Syed, Niveditha, & Ahmad, 2014). This same improvement has been revealed in our studies at pH 4.2.

Finally, Fig. 1B also shows the TFV release results of samples combining GG and ERS. This combination is the least studied, although the combination of GG and Eudragit RL (Elkhodairy et al., 2014) has

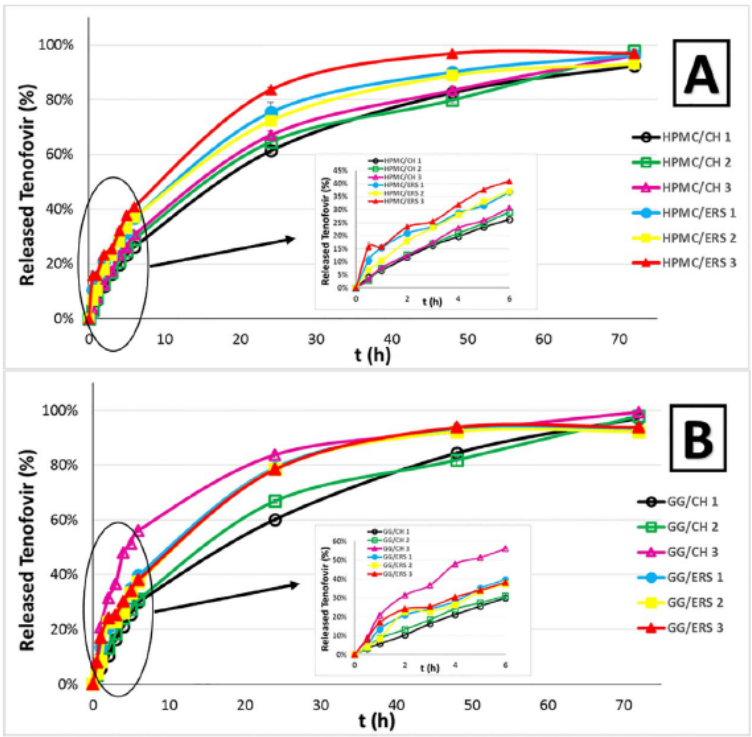

Fig. 1. Tenofovir release profiles obtained from tablets containing HPMC (A) or guar gum (B) in simulated vaginal fluid.

been tested, and is certainly the one that yields the least predictable results. All samples manufactured with the GG/ERS combination show better control over release compared to the profiles for the polymers separately (Notario-Pérez et al., 2017). The presence of ERS in the tablet matrix prevents initial drug loss (De Amit, Datta, & Mukherjee, 2011), indicating a synergic interaction between both polymers. However, although the combination of these polymers appears to be a good choice for the development of colonic release tablets (De Amit et al., 2011; Lai et al., 2010), the limited control over drug release enabled by these polymers compared to HPMC and CH renders them unsuitable for the design of our tablets for vaginal controlled release of TFV. These samples confirm that the combination of different polymers is an excellent way to improve the features offered by each one separately.

Therefore, based on the release studies, formulations combining CH and HPMC are the most suitable for the controlled release of TFV, as they offer sustained release of more than 90% of the drug over 72 h. Samples with CH and GG (if GG is in higher or equal ratio to CH) can also be considered for this purpose.

Similarity factor (f_2) was used to compare the experimental results. There were compared data from samples with the same polymers (in order to evaluate influence of polymers ratio) and from samples with the same ratio but different polymers (to evaluate the influence of polymer type). f_2 values obtained are represented in Table 2.

When comparing samples that combine HPMC and CH among them no significant differences are observed. It is the same with samples that combine GG and ERS in different ratios (Table 2). However, there is significant difference between GG/CH 3 sample and the other mixtures of these polymers (GG/CH 1 and GG/CH 2). This is due, as already mentioned, to the abrupt release of TFV that occurs in sample GG/CH 3. According to f_2 values, there is also no similarity between samples HPMC/ERS 2 and HPMC/ERS 3. As discussed above, in the combination of these two polymers no interaction is observed, so it is logical that those samples in which one of the polymers is in greater ratio differ from each other, since the polymers alone also have a very different behaviour (Notario-Pérez et al., 2017).

When analyzing the influence of the polymer on TFV release, it can be observed that there is no significant difference between samples combining HPMC/ERS and those with GG/ERS, regardless of the proportions in which they are combined (Table 2). This is due to the fact that the inclusion of ERS causes a rapid release of the TFV independently of the polymer with which it is combined. In contrast, there are differences between samples HPMC/CH and HPMC/ERS, HPMC/CH and GG/ERS, and also GG/CH and GG/ERS. The absence of similarity between these three polymers combinations shows statistically that CH clearly is able to control the release of TFV much better than ERS, whatever the polymer it combines with. Finally, it should be mentioned that the sample GG/CH 3 always shows a significant difference with the other samples, undoubtedly due to the already mentioned instability of the formulation.

The experimental results shown in Fig. 1 were fitted to the different mathematical model-dependent methods described above. After analysing each sample, the Higuchi, Korsmeyer-Peppas and Weibull were found to be the models with the best fit to the experimental results, since they showed the highest correlation coefficients (r^2) (Table 3).

The samples combining HPMC and CH or ERS and samples GG/CH 1 and GG/CH 2 have a good fit with the Korsmeyer-Peppas kinetic (Table 3). Since all the formulations studied in this paper are cylindrical tablets, a value of $n \leq 0.45$ means that TFV release follows a pure diffusion process (equivalent to the Higuchi model). A value of n between 0.45 and 0.89 indicates an anomalous transport where drug diffusion and the structural modification of the polymer matrix occur simultaneously. When n takes a value equal to 0.89, drug release occurs through transport case II; and if $n > 0.89$, through transport Supercase II. Both Case II and Supercase II involve the structural modification of the polymer matrix (Costa & Sousa Lobo, 2001; Dash et al., 2010). The n value in these samples indicates that they are all (except sample GG/CH 1) between 0.45 and 0.89, so the release of TFV is due to simultaneous mechanisms of structural modification and diffusion through the polymer matrix. Drug release is therefore conditioned by both the swelling and erosion of the formulation and its diffusion through the gel. Sample GG/CH 1 has a value of n over 0.89, meaning it follows a Supercase II drug release and implying extreme drug transport (Costa & Sousa Lobo, 2001). This sample has a similar drug release mechanism to CH when it is formulated as a single polymer (Notario-Pérez et al., 2017). The values of K_{KP} constant (Table 4) should also be mentioned, as the samples combining HPMC/ERS can be seen to have a much higher value than the other samples that fit this kinetic, suggesting that drug release occurs more rapidly in these formulations.

The same samples that best fit the Higuchi kinetic also fit the Korsmeyer-Peppas model (Table 3), implying that the drug release in these samples is due to a pure diffusion process. However, the Higuchi kinetic can only be applied when the swelling and dissolution of the matrix are negligible (Dash et al., 2010), and as the tablet can be observed to gel in all cases during the drug release test, this kinetic model is not applicable to these samples. The values of the Higuchi dissolution constant (K_H) are also similar to the K_{KP} values (Table 4). It is worth noting that sample GG/CH 3 does not fit this kinetic (or any other) (Table 3), because the combination in these proportions forms a very unstable gel that collapses rapidly.

Finally, the Weibull kinetic must also be mentioned, with which all the samples evaluated (except the aforementioned GG/CH 3) have a good fit (Table 3). The Weibull model is used to analyse release from matrix-type systems, and is the kinetic that best fit the ERS tablets (Notario-Pérez et al., 2017). In samples combining GG and ERS, this is the kinetic with the best fit to the drug delivery data, as in these samples there is rapid formation of a durable GG gel in which the ERS particles are intercalated, and the erosion of this gel is negligible while the TFV is being released. Since all samples fit this mathematical model, the values of the constant K_w–representing the drug release rate–allows the comparison of the rate at which drug release occurs across all the

Table 2
Similarity factors (f_2) values for the release profiles.

Reference	Problem	f_2
HPMC/CH 1	HPMC/CH 2	84.3
HPMC/CH 1	HPMC/CH 3	76.9
HPMC/CH 2	HPMC/CH 3	85.6
HPMC/ERS 1	HPMC/ERS 2	77.7
HPMC/ERS 1	HPMC/ERS 3	67.5
HPMC/ERS 2	HPMC/ERS 3	**60.6**
GG/CH 1	GG/CH 2	75.4
GG/CH 1	GG/CH 3	**34.5**
GG/CH 2	GG/CH 3	**37.2**
GG/ERS 1	GG/ERS 2	80.9
GG/ERS 1	GG/ERS 3	80.8
GG/ERS 3	GG/ERS 3	71.4
HPMC/CH 1	HPMC/ERS 1	**52.3**
HPMC/CH 1	GG/CH 1	85.0
HPMC/CH 1	GG/ERS 1	**49.9**
HPMC/ERS 1	GG/CH 1	**52.8**
HPMC/ERS 1	GG/ERS 1	76.1
GG/CH 1	GG/ERS 1	**50.8**
HPMC/CH 2	HPMC/ERS 2	**60.8**
HPMC/CH 2	GG/CH 2	83.8
HPMC/CH 2	GG/ERS 2	**56.0**
HPMC/ERS 2	GG/CH 2	67.7
HPMC/ERS 2	GG/ERS 2	75.7
GG/CH 2	GG/ERS 2	**60.6**
HPMC/CH 3	HPMC/ERS 3	**27.5**
HPMC/CH 3	GG/CH 3	**24.9**
HPMC/CH 3	GG/ERS 3	**28.0**
HPMC/ERS 3	GG/CH 3	**49.4**
HPMC/ERS 3	GG/ERS 3	71.7
GG/CH 3	GG/ERS 3	**47.2**

Values in bold are those lower than 65 (which means that there is significant difference).

Table 3
Correlation coefficients obtained when experimental data are fitted to the different mathematical models.

Sample	Correlation coefficients (r^2)						
	Zero order	First order	Higuchi	Hixson-Crowell	Hopfenberg	Korsmeyer-Peppas	Weibull
HPMC/CH 1	0.9340	0.6586	**0.9942**	0.9849	0.9753	**0.9994**	**0.9967**
HPMC/CH 2	0.9007	0.5836	**0.9879**	0.9609	0.9479	**0.9851**	**0.9815**
HPMC/CH 3	0.8997	0.5900	**0.9878**	0.9661	0.9522	**0.9911**	**0.9871**
HPMC/ERS 1	0.8805	0.7260	**0.9884**	0.9670	0.9494	**0.9929**	**0.9912**
HPMC/ERS 2	0.8726	0.6180	**0.9865**	0.9642	0.9455	**0.9990**	**0.9898**
HPMC/ERS 3	0.8639	0.7424	**0.9808**	0.9780	0.9559	**0.9935**	**0.9987**
GG/CH 1	0.9233	0.5895	**0.9917**	0.9822	0.9717	**0.9988**	**0.9932**
GG/CH 2	0.8876	0.5718	**0.9865**	0.9568	0.9420	**0.9835**	**0.9802**
GG/CH 3	0.6909	0.4455	**0.8969**	0.8702	0.8289	**0.9650**	**0.9380**
GG/ERS 1	0.8583	0.5523	**0.9795**	0.9635	0.9419	**0.9476**	**0.9923**
GG/ERS 2	0.8614	0.5309	**0.9768**	0.9618	0.9410	**0.9565**	**0.9905**
GG/ERS 3	0.8711	0.6467	**0.9860**	0.9735	0.9536	**0.9480**	**0.9962**

Bold values for the kinetics with the best fit.

Table 4
TFV release kinetics from the different samples showing the n, K_{KP}, K_H and K_W kinetic constants for the three models with the best fit.

Sample	Higuchi	Korsmeyer-Peppas		Weibull
	K_H	K_{KP}	n	K_w
HPMC/CH 1	0.125	0.068	0.76	0.035
HPMC/CH 2	0.124	0.064	0.86	0.032
HPMC/CH 3	0.129	0.067	0.85	0.036
HPMC/ERS 1	0.133	0.148	0.47	0.046
HPMC/ERS 2	0.134	0.107	0.69	0.043
HPMC/ERS 3	0.143	0.154	0.53	0.069
GG/CH 1	0.127	0.057	0.92	0.036
GG/CH 2	0.126	0.071	0.85	0.034
GG/CH 3	0.134	0.173	0.70	0.050
GG/ERS 1	0.142	0.104	0.77	0.053
GG/ERS 2	0.143	0.085	0.87	0.052
GG/ERS 3	0.139	0.141	0.57	0.055

samples. Sample HPMC/CH 2 can be seen to release the TFV in a more sustained manner, although closely followed by the remaining samples combining HPMC and CH, and GG/CH 1 and GG/CH 2.

3.2. Swelling test

Swelling capacity is a property of certain hydrophilic polymers that are able to capture water in their structure, causing a relaxation of the polymer chains and inducing a change from a crystalline to a rubbery state, forming a gel layer (Maderuelo, Zarzuelo, & Lanao, 2011). Since all the formulations evaluated contain at least one swellable polymer, the tablets capture aqueous medium in the presence of SVF. These polymers can be used for sustained drug delivery where the release mechanism is diffusion through the gel (Elzayat et al., 2016; Kulinowski et al., 2016).

In all instances the formulation swells rapidly in the early hours, and later continues to gain weight more slowly until the maximum degree of swelling is reached at between 48 and 96 h. The formulation then dissolves in a progressively accentuated manner. Swelling and erosion processes are therefore present in all cases. In Fig. 2 it can be observed the evolution of tablets aspect after immersion in SVF.

Table 5 contains a summary of the data on the swelling ratio of the samples. All samples combine a polymer with high swelling capacity (GG or HPMC) with another that hardly captures water (CH or ERS). As shown in the test, GG and HPMC have a high swelling capacity and capture a large volume of aqueous medium, which can make them uncomfortable for their clinical application (Aminabhavi, Nadagouda, Joshi, & More, 2014; Notario-Pérez et al., 2017; Yin et al., 2013). In contrast, chitosan has moderate swelling and ERS is a hydrophobic polymer that scarcely captures water (Agarwal and Murthy, 2015; Grund, Koerber, Walther, & Bodmeier, 2014; Kavianinia et al., 2016; Notario-Pérez et al., 2017). The formulation with the highest proportion of GG or HPMC will therefore always swell most, followed by the combination of both polymers in equal parts, and finally the one contains a higher proportion of the polymer that swells less (CH or ERS).

The results are somewhat predictable in the combinations of HPMC or GG with ERS, as ERS does not capture water so the SR depends only on the amount of HPMC or GG in the sample. However, in samples combining HPMC and CH the gel erosion rate can be seen to be lower, taking longer to completely erode than when HPMC is formulated alone (Table 5). Other authors have observed that free amino groups of CH protonate in acidic medium when formulated alone, increasing matrix swelling, while this erosion is retarded in the presence of other polymers (Elkhodairy et al., 2014). Moreover, sample HPMC/CH 3 lead to a better releasing TFV performance (as observed in Fig. 1), a phenomena that is due to CH lower capacity for water uptake (as observed in Table 5), and consequently the amount of the mixed gel formed by these two polymers in the presence of water will be smaller in the tablets with more CH, making easier the exit of TFV. In GG/ERS samples there is no change in the erosion rate compared to GG as a single polymer, although the formulation swells to a lower maximum swelling ratio due to the presence of ERS, preventing the early swelling of the formulation (De Amit et al., 2011). The complete erosion of the system is also completed in less time (Table 5). The samples containing HPMC and CH have a different swelling behaviour (Table 5): the swelling behaviour in sample HPMC/CH 2 is similar to HPMC alone, with a high initial swelling rate and an even higher maximum swelling ratio than HPMC, and a faster erosion rate; sample HPMC/CH 3 has a moderate swelling, more similar to CH, although the gel erodes much more slowly. Sample HPMC/CH 1, containing equal proportions of HPMC and CH, has an intermediate behaviour, since the maximum swelling rate is similar to HPMC, but the weight remains constant between 48 and 120 h, as occurs with CH tablets. Finally, in samples GG/CH it is worth noting the accelerated erosion of GG when combined with CH, as these samples take about 216 h to erode completely, in contrast with over 400 h for GG gel (Table 5).

Although the combination of GG and ERS seems to have an intermediate behaviour in the swelling test than for each polymer separately (Notario-Pérez et al., 2017), there is evidence of a possible interaction between them if these samples are compared with samples combining HPMC and ERS. While GG has a much higher maximum swelling ratio (SR_{max}) and area under the curve (AUC) than HPMC, HPMC and ERS combinations show higher values of these parameters than GG and ERS combinations.

The AUC is a parameter that combines the data of SR_{max} and t_∞. It can be observed that the value of AUC is lower in samples with more

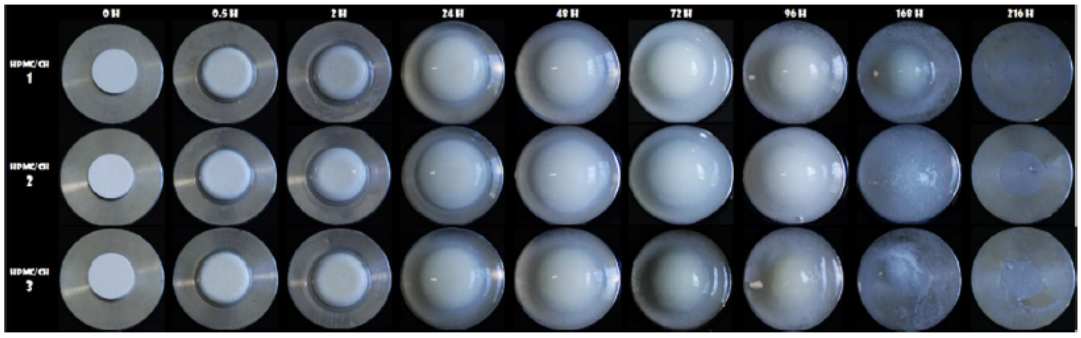

Fig. 2. Evolution of HPMC/CH 1, 2 and 3 matrices aspect after immersion in simulated vaginal fluid after 0.5, 2, 24, 48, 72, 96, 168 and 216 h.

Table 5
Summary of data obtained from the swelling test of tablets in simulated vaginal fluid, showing maximum swelling ratio (SR_{max}), time when the SR_{max} is reached (t_{SRmax}), time until complete erosion of the system (t_∞) and area under the curve between time 0 and complete erosion ($AUC_{0 \rightarrow t\infty}$). Data for compacted physical mixtures containing single polymers have been obtained from Notario-Pérez et al. (2017).

Sample	SR_{max} (± SD) (%)	t_{SRmax} (h)	t_∞ (h)	AUC
HPMC/CH 1	768 (± 13)	72	264	1412
HPMC/CH 2	989 (± 13)	72	264	1472
HPMC/CH 3	387 (± 12)	72	264	786
HPMC/ERS 1	582 (± 4)	96	384	1597
HPMC/ERS 2	709 (± 15)	72	408	1561
HPMC/ERS 3	415 (± 9)	72	384	989
GG/CH 1	707 (± 6)	72	264	1157
GG/CH 2	878 (± 2)	72	264	1197
GG/CH 3	498 (± 4)	72	264	724
GG/ERS 1	656 (± 8)	96	408	1331
GG/ERS 2	876 (± 13)	96	456	1856
GG/ERS 3	562 (± 27)	120	360	1222
*HPMC**	*892* (± 5)	*96*	*288*	*1799*
*CH**	*183* (± 17)	*48*	*288*	*579*
*GG**	*1328* (± 133)	*96*	*456*	*3391*
*ERS**	*79* (± 6)	*48*	–	–

CH, due to the lower SR_{max} of this polymer, and higher in samples with more GG, as a consequence of its high SR_{max} and longer t_∞. It should be mentioned that the inclusion of ERS, although it is a polymer that does not have the capacity to capture water, lengthens the AUC value more than the inclusion of CH, undoubtedly because the ERS prolongs the erosion time of the formulations.

Although the key criteria for selecting the optimum formulation are control over the drug release and mucoadhesion to vaginal mucosa, swelling must be taken into account in samples in which these characteristics are similar, as formulations capturing less water will be more comfortable. This is where the combinations containing a greater proportion of CH or ERS show their strength, as the lower uptake of water makes them easier for clinical application. In the case of sustained use of the treatment, the erosion of the system must be as fast as possible once the drug has been released so another tablet can be administered. From this standpoint, combinations containing CH would be the most suitable as they erode most rapidly. However, it should be noted that there is no need to wait for the complete erosion of the system before administering the next dose, as by the final days only a gel remains which would not hinder the insertion of a new tablet.

3.2.1. Electron microscopy micrographs

Complementary techniques used with prepared swelling witnesses allow a better understanding of the swelling behaviour of the formulations. The first of these is field-emission scanning electron microscopy (FE-SEM), used to create micrographs from swelling witnesses. The comparison of the micrographs from formulations combining HPMC and CH (Fig. 3B) reveals that when the proportion of HPMC is greater, the typical canal structure of this polymer can still be distinguished (Notario-Pérez et al., 2017). When the ratios are equal (Fig. 3A) or when CH is the majority polymer (Fig. 3C), the sponge structure typical of CH (Notario-Pérez et al., 2017) predominates. However, the presence of HPMC can be seen in the fact that the greater the proportion of this polymer, the larger the gaps in the formulation, due to HPMC's superior ability to capture water. The gaps inside the polymers are related to the swelling ratio, as when the gaps are larger, the formulations swell more.

The combination of HPMC and ERS produces formulations in which no defined polymer structure is observed. Only in Fig. 3E, when the proportion of HPMC is greater than ERS, can small ducts be noted, although unstructured by the presence of ERS. This is because ERS does not capture water, and its presence makes it difficult for HPMC to swell as usual. The grainy appearance in Fig. 3D and F is due to ERS (Notario-Pérez et al., 2017), and the presence of HPMC can scarcely be seen. This explains why the ability to control drug release is lower in formulations containing ERS, as the polymers do not have a homogeneous structure. Swelling behaviour is also related to this, as the formulation has higher swelling values when HPMC is the majority polymer, leading to the presence of small ducts that retain water.

Tablets combining GG and CH (Fig. 3G–I) have a very similar structure, regardless of the proportions in which the polymers are present. However, differences can be observed among the various proportions of the GG/CH mixture in the release and swelling test.

Finally, the analysis of the formulations combining GG and ERS shows that when the quantity of GG is higher than ERS (Fig. 3K), the sheets characteristic of GG can be seen (Notario-Pérez et al., 2017), but the ERS can be seen to be adhered to them. Fig. 3J, where GG and ERS are present in equal proportions, shows a similar structure, although in this case the amount of ERS is greater, and the ability to capture water is therefore lower. No defined structure is observed in Fig. 3L due to the majority presence of ERS. The swelling ratio is also greater when the formulation contains less ERS, and creating more free space between the sheets of GG. Another theory is that if the space between the sheets of GG is occupied by ERS, it is more difficult for the drug to diffuse through the polymer. This may explain why it takes longer to release the TFV from the dosage form when GG and ERS are mixed.

3.2.2. Porosity measurement

The same swelling witnesses have also been characterised by Hg porosimetry. Fig. 4 shows the pore size distributions (PSD) of these porous microstructures.

The most notable finding in Fig. 4 is that while a unimodal pore distribution was observed in the standard PSD obtained with a single polymer, the combinations cause, in most of the cases, a multimodal pore size distribution due to the contribution of polymers with different swelling behaviour to the pore formation. However, some combinations (HMPC/CH 2 and GG/CH 2) also show a unimodal distribution, undoubtedly because the higher proportion of HPMC or GG makes the

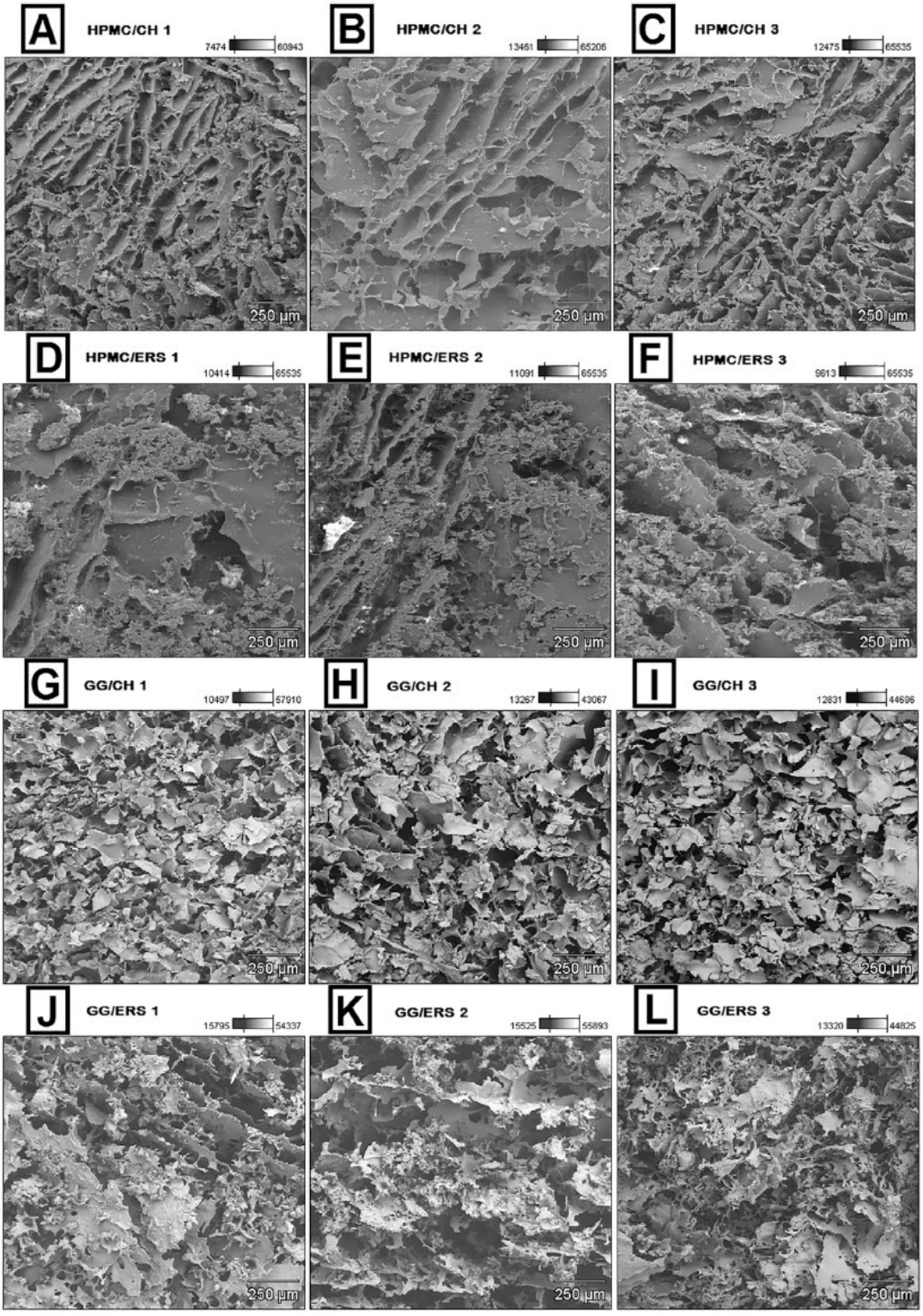

Fig. 3. Electron microscopy micrographs of swelling witnesses obtained from tablets. Accelerating voltage: 15.0 kV. Magnification: 60.

characteristic pores of these polymers predominate.

The results in Fig. 4A reveal that HPMC PSD predominates in the combinations of HPMC and CH, since the pores are around 100 µm, as occurs in the HPMC standard (Notario-Pérez et al., 2017). Pores between 50 and 0.1 µm, which predominate in CH, are scarce in these samples, similar to the HPMC standard. Nevertheless, it can be observed that the more CH the sample contains, the fewer and slightly smaller the pores. This similar PSD explains why drug release from these

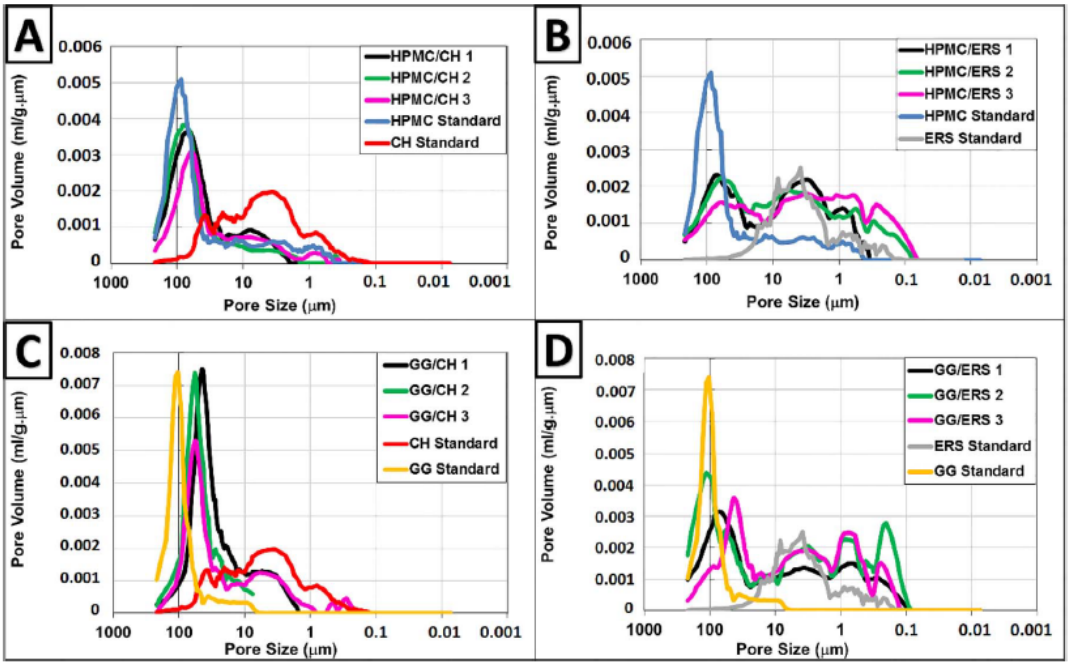

Fig. 4. Pore size distributions obtained from swelling witnesses in simulated vaginal fluid including HPMC and chitosan (A), HPMC and Eudragit® RS PO (B), guar gum and chitosan (C) and guar gum and Eudragit® RS (D), all compared with witnesses containing the polymer standard from Notario-Pérez et al. (2017).

samples follows a similar pattern. The fewer pores in combinations with a higher ratio of CH also accounts for the lower SR. Samples combining HPMC and ERS (Fig. 4B) have a well-distributed PSD, with pores between 100 and 0.1 µm contributing homogeneously to the total pore volume. This is significant, as HPMC and ERS have very different typical PSDs; while HPMC has mainly pores of around 100 µm, ERS pores are below 50 µm. The explanation is that there is no interaction between these two polymers in the combination. The fact that each polymer contributes its properties separately–so the result is a combination of both–can also be seen in the drug release, swelling and mucoadhesion tests, since they all show that the result of the samples combining the polymers is midway between the result obtained when evaluating the polymers separately.

It is noteworthy that the pores are smaller in the GG/CH samples (Fig. 4C)–between 100 and 50 µm–than for GG alone, where they are over 100 µm. However, in samples GG/CH 1 and GG/CH 2, these pores contribute the same volume as in GG. In contrast, these pores are present in much lower amounts in sample GG/CH 3, undoubtedly due to the aforementioned destructuring of the tablet. Finally, it should be noted that while the GG standard has no pores smaller than 10 µm, the combination contains pores of this size due to the presence of CH. It is interesting that the PSD obtained with samples GG/CH 1 and GG/CH 2 is similar to the PSD of the HPMC standard. This could explain why these two samples show a sustained release of TFV that is as good as in HPMC. Finally, Fig. 4D shows samples combining GG/ERS and, as occurs in the drug release and mucoadhesion tests, the results are unpredictable. These samples contain a large number of pores between 50 and 100 µm due to the presence of guar gum, as the more GG the sample contains, the closer the pore size to 100 µm. However, while the GG has no pores below 10 µm and the ERS has very few pores smaller than 1 µm, in the combinations of these polymers most of the pores are between 1 and 0.1 µm. This again suggests an interaction between the two polymers, which was already hinted after the results obtained in the previous tests.

Table 6 shows a summary of the results obtained in the Hg porosimetry of the swelling witnesses. The pore volume (Vp) is directly related to the swelling capacity of the polymers. This is because the water forming the gel is removed during freeze-drying and the pores detected correspond to the space occupied by the water in the gel. Samples containing GG therefore have the highest Vp values, and samples with ERS the lowest. The pore area (Sp) appears to be related to the size of the pores, since Sp values are lower when CH is present due to the smaller pore size common in this polymer; while samples combining HPMC and CH, which show better control over TFV release, have the smallest Sp of all the samples, hindering the drug diffusion through the gel. Finally, it should be noted that porosity (P) values are directly related to Vp and mean pore size (Dp). Sample GG/CH 3 stands out from the rest for its much higher apparent density (ρ_A), and for having the largest porosity due to the disruption suffered during swelling.

Table 6
Pore volume (Vp), pore area (Sp), mean pore size (Dp), apparent density (ρ_A) and porosity (P) of HPMC, CH, ERS and GG witnesses.

Witness	Vp ($cm^3\ g^{-1}$)	Sp ($m^2\ g^{-1}$)	Dp (µm)	ρ_A ($cm^3\ g^{-1}$)	P (%)
HPMC/CH 1	5.64	0.41	71.68	0.86	82
HPMC/CH 2	5.17	0.30	84.89	1.02	84
HPMC/CH 3	3.97	0.32	66.29	1.15	82
HPMC/ERS 1	4.40	0.95	60.46	1.02	81
HPMC/ERS 2	4.58	0.68	58.17	0.97	81
HPMC/ERS 3	3.47	0.66	55.99	1.00	77
GG/CH 1	7.33	0.82	43.50	0.98	87
GG/CH 2	6.97	0.56	57.49	1.00	75
GG/CH 3	5.18	0.50	56.52	2.96	93
GG/ERS 1	5.42	0.65	70.90	0.90	83
GG/ERS 2	6.86	0.81	97.84	0.97	86
GG/ERS 3	4.93	0.82	45.09	0.99	83

3.3. X-ray diffraction

The analyses made by X-ray diffraction have allowed to characterise the materials used in the preparation of the tablets. The results allow us

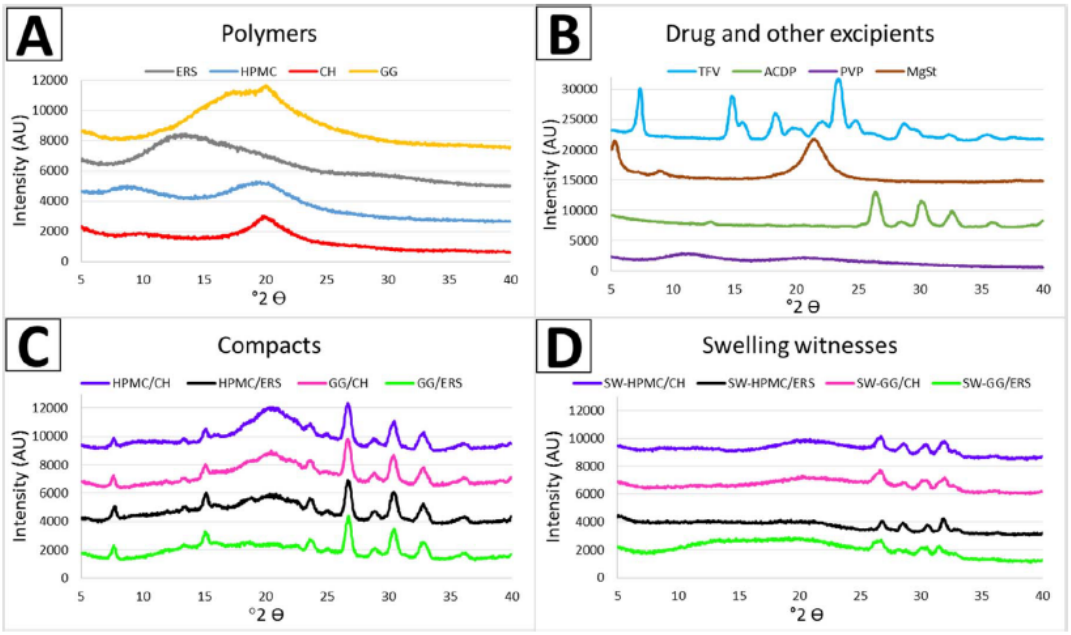

Fig. 5. X-ray diffraction patterns of HPMC, CH, ERS and GG (A), TFV, ACDP, PVP y MgSt (B), tablets from samples HPMC/CH 1, HPMC/ERS 1, GG/CH 1 and GG/ERS 1 (C) and swelling witnesses obtained from these same samples (D).

to state that all the polymers used are amorphous in nature (Fig. 5A). TFV is a drug with crystalline character, with characteristic peaks at 7.3, 14.7, 18.3 and 23.4° 2Θ (Fig. 5B). Concerning the other excipients used, PVP is amorphous while MgSt and ACDP are crystalline (Fig. 5B).

In Fig. 5C it is observed how the characteristic peaks of ACDP (four peaks between 26 and 33° 2Θ) can be clearly seen in the analysis of the prepared tablets. In addition, an amorphous halo is observed between 18 and 22°, with greater intensity the more HPMC and CH the sample contains. The characteristic peaks of TFV are still appreciated, although the 18.3° peak is masked by the signal of the polymers.

In contrast, Fig. 5D – where the swelling witnesses are analysed- indicates that the structure of the tablets is modified after their immersion in SVF. On the one hand, it can be seen how TFV peaks have disappeared completely in the witnesses, because the drug has been released and is no longer present in the analysed samples. It is also observed that the amorphous halo corresponding to the polymers has markedly decreased their intensity, undoubtedly because they have been hydrated and their structure has completely changed. However, it should be noted that in all formulations the characteristic peaks of ACDP (between 26 and 33°) are perfectly observed, since it acts as a structural agent and remains in the analysed samples.

3.4. Mucoadhesion test

Differences in the dwelling time of the formulations attached to the vaginal mucosa can be observed in the mucoadhesion test (Fig. 6). Samples combining HPMC and ERS show high residence time adhered to the mucosa–over 150 h in all cases–and also indicate that the higher the amount of HPMC, the greater the mucoadhesion (HPMC/ERS 2 > HPMC/ERS 1 > HPMC/ERS 3). Both polymers have good mucoadhesive properties separately (Notario-Pérez et al., 2017), so it is logical that their combination should also remain attached for longer. The next combination of polymers remaining attached for longest is GG/CH, always over 96 h, although when the proportion of GG is equal to or greater than CH (GG/CH 1 and GG/CH 2), mucoadhesion time extends to over 120 h. Sample GG/CH 3 has lower mucoadhesion time, possibly due to its higher proportion of CH, which is less mucoadhesive than GG, or to the faster breakdown of the gel, as observed in the drug release and swelling results. The combinations HPMC/CH remain attached to the vaginal mucosa for about 96 h, regardless of the polymer ratio. When CH is combined with HPMC or GG, the adhesion of the CH is caused rather by the effect of the mucoadhesive nature of these polymers–which bind via hydrogen bonds–than by the interaction between the charges. Finally, the most striking results can be found in the GG and ERS combinations. Although separately both polymers have a high mucoadhesive power, remaining attached for 162 and 140 h respectively (Notario-Pérez et al., 2017), when combined they deliver residence times of between 65 and 80 h in the mucoadhesion test. This result suggests a possible physical interaction between these two polymers.

In view of these results, we can report that all combinations of polymers except GG and ERS would be useful for developing vaginal mucoadhesive tablets for sustained release of TFV, as they remain attached to the mucosa for at least 96 h, enough for the release of the entire drug included in the formulation. Nevertheless, for the comfort of the patients and with a view to subsequent administrations, it would be advisable to choose the formulations that remain less time in the vaginal environment after 96 h have elapsed. Thus the HPMC/CH samples would be the most suitable, although samples combining GG and CH also perform well.

Once all tests have been performed, the most suitable formulations must be selected for possible *in vivo* HIV prevention. The results of each sample in the assays are summarised in Table 7. Drug release results are compared using K_w values obtained when the curves in Fig. 1 are fitted to the Weibull model, since all samples fit well to this kinetic. A K_w lower than 0.04 is considered excellent (✓✓), a value between 0.04 and 0.049 is considered acceptable (✓) and when K_w is higher than 0.049 it is considered unacceptable (x). Mucoadhesion results are unacceptable (x) when the sample is unable to remain attached for at least 72 h (3 days), acceptable (✓) when this time is reached, and excellent (✓✓) if the dwelling time in the vaginal mucosa is between 72 and 96 h (which is the time until complete TFV release is achieved), as this ensures that it only remains attached for the time the drug is being released. Finally, swelling test results are unacceptable (x) with SR values over 500%, as these formulations would not be comfortable for women. Samples are considered acceptable (✓) if SR is never over 500%, and excellent (✓✓)

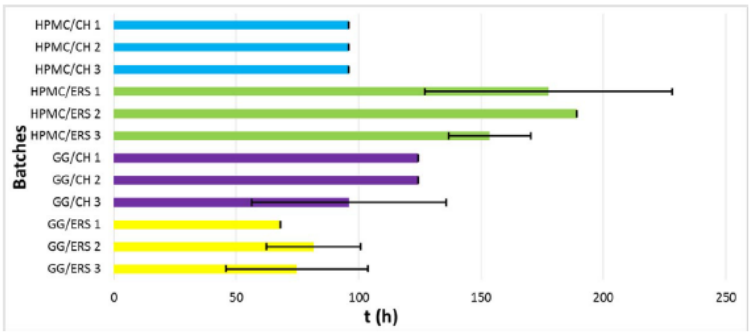

Fig. 6. Mucoadhesion residence time of Tenofovir tablets in simulated vaginal fluid.

Table 7
Comparison of the different samples with a summary of the results obtained in the tests.

Sample	Drug release	Mucoadhesion	Swelling
HPMC/CH 1	✓✓	✓✓	✗
HPMC/CH 2	✓✓	✓✓	✗
HPMC/CH 3	✓✓	✓✓	✓✓
HPMC/ERS 1	✓	✓	✗
HPMC/ERS 2	✓	✓	✗
HPMC/ERS 3	✗	✓	✓
GG/CH 1	✓✓	✓	✗
GG/CH 2	✓✓	✓	✗
GG/CH 3	✗	✓✓	✓✓
GG/ERS 1	✗	✗	✗
GG/ERS 2	✗	✓✓	✗
GG/ERS 3	✗	✓✓	✗

if the gel also erodes completely in less than 300 h, since this ensures that it does not take excessive time to gel completely and the next tablet can be inserted.

Samples combining HPMC and CH and samples GG/CH 1 and GG/CH 2 are excellent in terms of sustained drug release of TFV and mucoadhesion to vaginal mucosa. Samples with HPMC and CH also show excellent bioadhesion to the vaginal mucosa. We selected sample HPMC/CH 3 as the optimal formulation as its swelling profile makes it more appropriate for use in women.

4. Conclusions

The above results point to the conclusion that the combination of different polymers in the same formulation, in the right proportion, can enhance the advantages offered by each one individually.

Data obtained allow to conclude that the combination of HPMC and CH in tablets results in robust formulations which are the most suitable for: a) the prolonged vaginal release of TFV (72 h) and b) vaginal mucoadhesion residence time (96 h). The formulation containing a higher proportion of CH than HPMC has a moderate swelling profile and fast erosion of the formed gel, making it more comfortable for clinical application. By including the anti-HIV drug TFV, this formulation administered vaginally may be useful in preventing the sexual transmission of the virus from men to women.

Authors' contribution

Roberto Ruiz-Caro, Aitana Tamayo, Juan Rubio and María-Dolores Veiga designed and planned the experiments. Fernando Notario-Pérez, Araceli Martín-Illana and Raúl Cazorla-Luna conducted the experiments. All authors have approved the final article. María-Dolores Veiga is the senior author and project leader.

Conflict of interest

The authors declare no conflict of interest.

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CAPÍTULO IV

IMPROVEMENT OF TENOFOVIR VAGINAL RELEASE FROM HYDROPHILIC MATRICES THROUGH DRUG GRANULATION WITH HYDROPHOBIC POLYMERS

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Improvement of Tenofovir vaginal release from hydrophilic matrices through drug granulation with hydrophobic polymers

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ABSTRACT

Sustained-release vaginal microbicides hold out great hope for the prevention of sexual transmission of HIV from men to women. Tenofovir (TFV) –an antiretroviral drug– sustained-release vaginal compacts combining two release control systems (by drug-loading granules with hydrophobic polymers and incorporating them in a hydrophilic matrix) are proposed in this work as a possible microbicide. The polymers used for the drug granules are Eudragit® RS (ERS), an acrylic derivative, and Zein, a maize protein. The hydrophilic matrix is composed of a mixture of hydroxypropylmethyl cellulose (HPMC) and chitosan (CH). The thermal, microscopic, spectrophotometric and X-ray diffraction analysis showed that the drug was not altered during the granulation process. Studies of TFV release, swelling and *ex vivo* mucoadhesion were subsequently performed on simulated vaginal fluid. The formulation whereby TFV is granulated using twice its weight in ERS, and then including these granules in a matrix in which the CH predominates over HPMC, allows the sustained release of TFV for 144 h, mucoadhesion to the vaginal mucosa for 150 h and a moderate swelling, making it the most suitable formulation of all those studied. These compacts would therefore offer women protection against the sexual acquisition of HIV.

1. Introduction

In recent decades, significant efforts have been made to develop a vaginal microbicide to protect women against the sexual acquisition of the human immunodeficiency virus (HIV) (Antimisiaris and Mourtas, 2015; Doggett et al., 2015). However, the main problem with formulations capable of reducing the incidence of infection is low adherence by women to prophylactic treatment (Friend and Kiser, 2013; Gengiah et al., 2014; Marrazzo et al., 2015; van der Straten et al., 2016). It has been demonstrated that the protection offered by microbicides that have shown efficacy in phase III clinical trials varies greatly depending on the frequency of use. For example, in the CAPRISA 004 trial it was observed that the protection of the formulation varied from 28% to 54% (Abdool Karim et al., 2010; Kashuba et al., 2015; McConville et al., 2014) depending on the adherence to use, and in the ASPIRE study, infection was reduced by 27%, although when data from regions with low adherence were excluded, the percentage rose to 37% (Baeten et al., 2016). The main aim of the formulations being developed today is therefore to improve the adherence of their predecessors. To this end, the goal is to develop microbicides capable of releasing the drug over several days in order to decrease the frequency of administration. Another factor that must also be improved is women's comfort (Domanska and Teitelman, 2012; Eakle et al., 2015; Woodsong and Holt, 2015).

Nowadays there are multiple resources available to obtain extended release systems. The release rate can be controlled by increasing the particle size of the drug or by forming insoluble crystals (Chogale et al., 2016). Another notable possibility is to produce microspheres, microcapsules or microgranules in which the drug is coated with a slow-dissolving polymer; these particles are subsequently used to obtain the appropriate dosage form (tablets, capsules…) (Rambhia and Ma, 2015; Wang et al., 2013). Release can also be controlled through the design of the pharmaceutical form. Some examples are insoluble matrices (capable of controlling drug diffusion) (Bouman et al., 2016; Shojaee et al., 2016), soluble matrices (in which the drug is slowly dissolved) (Cevher et al., 2014; Maderuelo et al., 2011), coated systems (where drug delivery is controlled by a cover through which it diffuses) (Guo and Shi, 2009), and osmotic systems (in which a semipermeable membrane

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allows the water to enter and dissolve the drug, which later emerges through a small orifice) (Rastogi et al., 2013).

Hydrophilic polymers able to form a gel in an aqueous medium have been shown to be an excellent choice for the sustained release of drugs in the vaginal environment (Baloğlu et al., 2006; Sharma et al., 2006). Pharmaceutical forms can be obtained with these characteristics and developed with numerous polymers, such as hydroxypropylmethyl cellulose (HPMC) (Baloğlu et al., 2006; Hani et al., 2016; Karasulu et al., 2004; Sánchez-Sánchez et al., 2015), chitosan (CH) (El-Kamel et al., 2002; Sánchez-Sánchez et al., 2015; Szymańska et al., 2014), pectin (Baloğlu et al., 2003, 2006), guar gum (Hani et al., 2016), xanthan gum (Hani et al., 2016), alginates (El-Kamel et al., 2002; Gavini et al., 2002) and carragenates (Sánchez-Sánchez et al., 2015). The results obtained in previous studies show that the combination of HPMC and CH appears to offer the best results due to its ability to control the release of water-soluble drugs and its high mucoadhesive capacity (Notario-Pérez et al., 2018).

The most frequently used polymers for obtaining functional coatings are insoluble but permeable in aqueous medium, such as acrylic derivatives or Zein. Worth highlighting among the acrylic derivatives are those marketed as Eudragit®, comprising a wide variety of polymethacrylate-based copolymers specifically designed to obtain functional coatings, and which modify their characteristics according to the pH of the target area for the drug release (Thakral et al., 2013). Eudragit® RS (ERS) and Eudragit® RL, whose structure includes quaternary ammonium groups and are permeable but insoluble in water, are the best choice for drug release in the vaginal environment (pH 4.2) (Akhgari and Tavakol, 2016; Thakral et al., 2013). As mentioned, coatings can also be formed with Zein, a maize protein consisting mainly of hydrophobic and neutral amino acids, which is pH-stable, insoluble in water, but soluble in alcoholic solutions (Karthikeyan et al., 2012; Zhang et al., 2016). Its use for the sustained release of drugs has lately been extended to include coatings and microparticles and capsules or implants made with this polymer (Bouman et al., 2016; Karthikeyan et al., 2012; Zhang et al., 2016).

Although the most common application of these polymers is for the production of functional coatings, mainly in coated tablets, they can potentially be used to granulate drugs thanks to their adhesive properties which enable granules to be obtained in which the drug is mixed with the hydrophobic polymer. The aim of this work is to produce microgranules of tenofovir (TFV), an antiretroviral drug, combined with ERS or Zein, for their subsequent inclusion in a hydrophilic polymer matrix made from HPMC and CH, two polymers capable of forming a gel in the presence of vaginal fluid, to develop TFV sustained-release vaginal tablets.

2. Materials and methods

2.1. Materials

Tenofovir (TFV, lot: FT104801401) was supplied by Carbosynth Limited (Berkshire, UK). Eudragit RS® (ERS; lot: G120238035) was supplied by Evonik (Essen, Germany). Zein (lot: SLBL9380V) was acquired from Sigma-Aldrich (St. Louis, MO, USA). Chitosan, with 97% deacetylation and a viscosity of 92 mPa·s (CH, lot: 8826900003), was provided by Nessler (Madrid, Spain). Hydroxypropylmethyl cellulose – Methocel® K 100 M (HPMC; lot: DT352711) was kindly supplied by Colorcon Ltd. (Kent, UK). Anhydrous calcium hydrogen phosphate – Emprove® (ACDP; lot: K93487944416) was supplied by Merck (Darmstadt, Germany). Polyvinylpyrrolidone – Kollidon® 30 (PVP; lot: 98-0820) was purchased from BASF Aktiengesellschaft (Ludwigshafen, Germany). Magnesium stearate PRS-CODEX (MgSt; lot: 85269 ALP) was acquired from Panreac (Barcelona, Spain).

All other reagents used in this study were of analytical grade and used without further purification. Demineralized water was used in all cases.

Table 1
Composition of granules (content in mg per each 30 mg of drug).

Granulate	TFV	ERS	Zein
TFV1E2	30	60	
TFV1E1	30	30	
TFV2E1	30	15	
TFV1Z2	30		60
TFV1Z1	30		30
TFV2Z1	30		15

2.2. Preparation of the granules

TFV granules were prepared with ERS or Zein, with different drug/polymer ratios. This was done by mixing the powdery components, then adding sufficient ethanol to form a mass, which was possible because both ERS and Zein are soluble in ethanol and their adhesiveness eliminates the need for a binding agent. Finally, this mass was passed through a 1 mm mesh and the resulting granulate was dried at room temperature for 24 h to ensure complete evaporation of the ethanol. The granules obtained are shown in Table 1.

2.3. Characterization of the granules

2.3.1. X-ray diffraction

The X-ray diffraction patterns of pure materials and all granulated systems were recorded by using an automated Philips® X'Pert-MPD X-ray diffractometer with Bragg–Brentano geometry. Samples were irradiated with monochromatized Cu-Kα radiation ($\lambda = 1.5406$ Å) at 45 kV, 40 mA and a time per step of 2 s, and analysed between 2θ angles of 5° and 50°.

2.3.2. Infrared spectroscopy

The pure materials and prepared granules were characterised by Fourier transform infrared attenuated total reflection spectroscopy (FTIR-ATR). FTIR-ATR spectra were obtained with a Perkin-Elmer spectrophotometer instrument equipped with a MIRacle™ accessory designed for ATR measurements (Perkin-Elmer, USA).

2.3.3. Thermal analysis

Differential scanning calorimetry (DSC) and thermogravimetric analysis (TGA) were performed in a SDT-Q 600 TA instruments TG/DTA analyser. About 5–10 mg of sample were placed in a pinholed aluminium sample pan with a lid and heated in atmospheric air to between 25° and 500 °C.

To perform hot stage microscopy (HSM), about 1 mg of sample was placed on a microscopic slide with a cover and heated at a rate of 2 °C/min on a Kofler stage. All samples were studied at between 30° and 350 °C. Microscopic examinations were made using a Thermogalen microscope fitted with the Kofler stage.

2.4. Preparation of the compacts

Batches of compacts were manufactured with a combination of hydrophilic polymers (HPMC and CH) containing the previously developed granules. Each granulated system was included in three different combinations of HPMC and CH (C1, with a ratio of 1:1 HPMC/CH; C2, with a ratio of 1.9:1 HPMC/CH; and C3, with a ratio of 1:1.9 HPMC/CH). The polymer combination was prepared by mixing HPMC, CH and ACDP, then wetting the mixture with a solution of PVP in ethanol to produce a mass, which was granulated using a 1 mm mesh and dried at room temperature for 24 h. Three granulates were obtained (C1, C2 and C3). Finally, each granulate was mixed with a sufficient amount of the TFV granules to ensure a content of 30 mg of drug and 290 mg of hydrophilic polymer per compact. MgSt was added to the total dried granulate before compaction. The final content of each batch

Table 2
Composition (mg/unit) of compacts.

Batch	HPMC	CH	ACDP	PVP	TFV	ERS	Zein	MgSt
TFV1E2 C1	145	145	45	27	30	60		3
TFV1E2 C2	190	100	45	27	30	60		3
TFV1E2 C3	100	190	45	27	30	60		3
TFV1E1 C1	145	145	45	27	30	30		3
TFV1E1 C2	190	100	45	27	30	30		3
TFV1E1 C3	100	190	45	27	30	30		3
TFV2E1 C1	145	145	45	27	30	15		3
TFV2E1 C2	190	100	45	27	30	15		3
TFV2E1 C3	100	190	45	27	30	15		3
TFV1Z2 C1	145	145	45	27	30		60	3
TFV1Z2 C2	190	100	45	27	30		60	3
TFV1Z2 C3	100	190	45	27	30		60	3
TFV1Z1 C1	145	145	45	27	30		30	3
TFV1Z1 C2	190	100	45	27	30		30	3
TFV1Z1 C3	100	190	45	27	30		30	3
TFV2Z1 C1	145	145	45	27	30		15	3
TFV2Z1 C2	190	100	45	27	30		15	3
TFV2Z1 C3	100	190	45	27	30		15	3

is shown in Table 2.

In all cases the compacts were prepared with a press similar to the one used for preparing solid samples for analysis by IR spectroscopy. 5 t of constant pressure was applied using a punch for 4 min. The manufactured compacts were cylindrical in shape and measured 13 mm in diameter and 2.5–2.8 mm in height.

2.5. Assessment of the compacts

2.5.1. Swelling behaviour

The swelling behaviour of the compacts in simulated vaginal fluid (SVF) (Owen and Katz, 1999) was analysed using the method described by Ruiz-Caro and Veiga-Ochoa (2009). Each analysis was done in a thermostatized shaking water bath (Selecta® UNITRONIC320 OR, Barcelona, Spain) at 37 °C and 15 opm and tested in triplicate. At given times the discs were removed from the medium, placed on filter paper to eliminate the excess liquid and weighed. The swelling ratio (SR) was calculated according to Eq.1:

$$SR = \left(\frac{Cs - Cd}{Cd}\right)\cdot 100 \quad (1)$$

where *Cs* and *Cd* correspond to the swollen and dry compact weights respectively.

Since ERS and Zein do not swell, the initial weight of the compacts is not constant in all batches but varies depending on the amount of these polymers in each batch. The adjusted swelling ratio (ASR) was therefore determined to compare the SR of different batches, where the weight increment is related to the amount of swellable polymer, as expressed in Eq.2:

$$ASR = \frac{SR\cdot Cd}{SP} \quad (2)$$

where *SR* is the swelling ratio determined by Eq.1, *Cd* is the dry compact weight and *SP* is the amount of swellable polymer in the compact.

2.5.2. Release study

The TFV release behaviour in each batch was evaluated with the method described by Sánchez-Sánchez et al. (2015). The samples were placed in a borosilicate glass bottle with 80 mL of the SVF and then in a thermostatized shaking water bath (Selecta® UNITRONIC320 OR, Barcelona, Spain) at 37 °C and 15 opm. The solubility of TFV in SVF, measured at room temperature, was established at 4 mg/mL, which ensures that release tests are performed in sink conditions. At given times, 5 mL samples were taken and filtered, and the medium was replaced with the same volume of SVF at the same temperature, in order to maintain the volume of SVF constant. TFV concentrations were quantified by UV spectroscopy at a wavelength of 260 nm in a Shimadzu® UV-1700 spectrophotometer (Kyoto, Japan). The test was performed in triplicate in each case.

The drug release experimental data were fitted to different model-dependent methods (zero order, first order, Higuchi, Hixson-Crowell, Hopfenberg, Korsmeyer-Peppas and Weibull models) to investigate the drug release kinetics (Costa and Sousa Lobo, 2001; Dash et al., 2010; Mamani et al., 2012).

The zero order model is applied to dosage forms that remain unchanged while the drug is released in a slow manner, and is represented by Eq.3, where Q_0 is the drug included in the formulation, Q_t is the amount of drug released at time t and K_0 is the zero order release constant.

$$Q_0 - Q_t = K_0\cdot t \quad (3)$$

The first order model represents the drug release as a function of surface action, and is usually applied to porous matrices. It is summarized as Eq.4, where Q_t is the drug released at time t, Q_0 is the total amount of drug and K_1 is the first order rate constant.

$$\log Q_t = \log Q_0 - \frac{K_1\cdot t}{2.303} \quad (4)$$

The Higuchi model is useful for matrix tablets, since it represents a diffusion mechanism based on Fick's law for drug release. The Higuchi equation is shown in Eq.5, where Q_t is the amount of drug released at time t and K_H is the Higuchi dissolution constant.

$$Q_t = K_H t^{1/2} \quad (5)$$

The mathematic model described by Hixson and Crowell can be applied to dosage forms where drug release takes place in parallel planes, and the dimension of the dosage form is reduced proportionally but its size remains constant. It is expressed as Eq.6, where Q_0, Q_t and K_{HC} are the initial amount of drug, the drug released at time t and a constant which includes the surface-volume relation respectively.

$$Q_0^{1/3} - Q_t^{1/3} = K_{HC}\cdot t \quad (6)$$

The Hopfenberg model is applied to surface-eroding dosage forms and is expressed as Eq.7, where Q_t is again the drug released at time t and Q_0 the total drug included. K_{HF} is the Hopfenberg rate constant, which includes the expression K_0/C_0a_0, where K_0 is the erosion rate constant, Q_0 is the initial drug concentration in the dosage form, and a_0 is half the initial system thickness. n_H is the Hopfenberg exponent, which is related to the geometry and has a value of 1 (for slab), 2 (for cylindrical) or 3 (for spherical) depending on the system.

$$\frac{Q_t}{Q_0} = 1 - [1 - k_{HF}\cdot t]^{n_H} \quad (7)$$

For our compacts, this expression can be summarized in Eq.8:

$$1 - \sqrt{Q_0 - Q_t} = k_{HF}\cdot t \quad (8)$$

The Weibull model is applied to matrix-type systems and is expressed as Eq.9, where Q_t and Q_0 are the drug released at time t and the total amount of drug included in the formulation respectively. t_{lag} is the lag time, a is a parameter that describes dependence on time, and b is a parameter that represents the shape of the dissolution profiles.

$$Q_t = Q_0\left[1 - e^{-\frac{(t-t_{lag})^b}{a}}\right] \quad (9)$$

Our experimental data, where t_{lag} has a value of zero and the curve has an exponential shape ($b = 1$), is summarized in Eq.10, where K_w represents the Weibull constant, corresponding to $1/a$.

$$\ln\left(1 - \frac{Q_t}{Q_0}\right) = -K_W\cdot t \quad (10)$$

Finally, the Korsmeyer-Peppas model describes the drug release as a function of time, as expressed in Eq.11, where Q_t/Q_∞, K_{KP} and n are the fraction of drug released at time t, a constant which includes the geometric and structural characteristics of the system and the release exponent respectively.

$$Q_t/Q_\infty = K_{KP} \cdot t^n \quad (11)$$

In this model the drug release mechanism is defined according to the value of n. Thus in our case, where the systems have the shape of cylindrical compacts, according to the value of n, TFV release would follow a pure diffusion process ($n \leq 0.45$), an anomalous transport where diffusion and structural modification occur simultaneously ($0.45 < n < 0.89$), transport case II ($n = 0.89$) or transport Supercase II ($n > 0.89$). Case II and Supercase II imply a structural modification of the polymer matrix.

2.5.3. Mucoadhesion test

Ex vivo mucoadhesion tests were performed according to the method described by Notario-Pérez et al. (Notario-Pérez et al., 2017). To determine how long the compact remained adhered to the vaginal mucosa, a sample of freshly excised veal vaginal mucosa (obtained from a local slaughterhouse) was fixed with an acrylic adhesive to an 8.5 cm × 5 cm stainless steel plate, and each compact was then adhered to the mucosa, applying a pressure of 500 g for 30 s. The system was placed inside a beaker containing 150 mL of SVF at an angle of 60°, then in the thermostatized shaking water bath (Selecta® UNITRONIC320 OR, Barcelona, Spain) at 37 °C and 15 opm. All batches were tested in duplicate, and the bioadhesion time of each batch was established as the average time it took for the compacts to be detached from the mucosa.

3. Results and discussion

3.1. Characterization of the granules

Multiple parameters of the drug properties, such as crystallinity, hydrophilicity/hydrophobicity and thermal properties, influence the release of the drug from the pharmaceutical dosage form and its therapeutic effectiveness (Tamayo et al., 2017). These characteristics can be modified during the preparation of TFV granules with ERS or Zein, making it essential to obtain a rigorous characterization.

3.1.1. X-ray diffraction

X-ray diffraction analysis clearly indicates that the TFV raw material is a crystalline substance with its principal peaks at 7.38, 14.78, 18.3 and 23.42° 2θ (Fig. 1). These findings are similar to those described by other authors (Agrahari et al., 2015). In contrast, ERS is amorphous, since it displays a diffuse background in its diffraction spectrum and its only outcome is a halo between 10 and 20° 2θ (Fig. 1A). Zein is also an amorphous substance (Huang et al., 2013), with two small intensity halos in its diffractogram (Fig. 1B).

Table 3
Intensity (expressed in AU) of the characteristic TFV peaks (° 2θ) of the different samples analysed.

	2θ			
Sample	7.38	14.78	18.30	23.42
TFV	9099	7852	5023	10,695
TFV2E1	6324	6503	4073	8671
TFV1E1	5312	4532	2480	4329
TFV1E2	5026	5090	3533	4766
TFV2Z1	7079	5850	3933	7610
TFV1Z1	4183	3377	2596	4531
TFV1Z2	4030	3151	2736	4045

As can be seen in Fig. 1A, the same characteristic peaks of the drug are obtained for the granules prepared by combining TFV and ERS, although the peaks decline in intensity as the TFV in the sample decreases. It should be noted that the peaks in the TFV1E2 sample have a higher intensity at 14.78°, 18.3° and 23.42° 2θ than the TFV1E1 sample (Table 3). This is because these peaks are in the area where the characteristic halo of the ERS is located, so when the content of this polymer is higher, the intensity of these granules increases. Exactly the same occurs with the granules combining TFV and Zein, since the intensity also decreases when the drug content is lower (Fig. 1B and Table 3). In this case, only the peak at 18.3° 2θ overlaps with the characteristic halo of Zein, and thus shows greater intensity for batch TFV1Z2 than TFV1Z1.

Therefore, in addition to identifying the crystalline character of the drug and the amorphous state of both polymers, the X-ray diffraction technique indicates there is no interaction between drug and polymer during the preparation of the granules.

3.1.2. Infrared spectroscopy

The spectroscopic characterization of the prepared granules and the raw materials used by ATR-FTIR is shown in Fig. 2. The analysis of the raw materials reveals the spectra for the drug (TFV) and the polymers (ERS and Zein). Tenofovir has a N—H stretching band at around 3200 cm^{-1}, while C—H stretching is observed at around 3100 cm^{-1}. However, the main TFV peak (outside the fingerprint region) is found at 1700 cm^{-1} and can be associated to the NH_2 group (Ramkumaar et al., 2013).

The prepared granules show a very similar spectrum in all combinations irrespective of the polymer/drug ratio used in their preparation. A combination of the peaks obtained when the pure substances are analysed separately can be seen in the spectra for the ERS and TFV mixtures (Fig. 2A). This polymer has a characteristic band of carbonyl groups at around 1700 cm^{-1}, coinciding with one of the characteristic bands of TFV. The main band of the granules is observed at this wavelength.

The same can be seen in granules combining TFV and Zein (Fig. 2B).

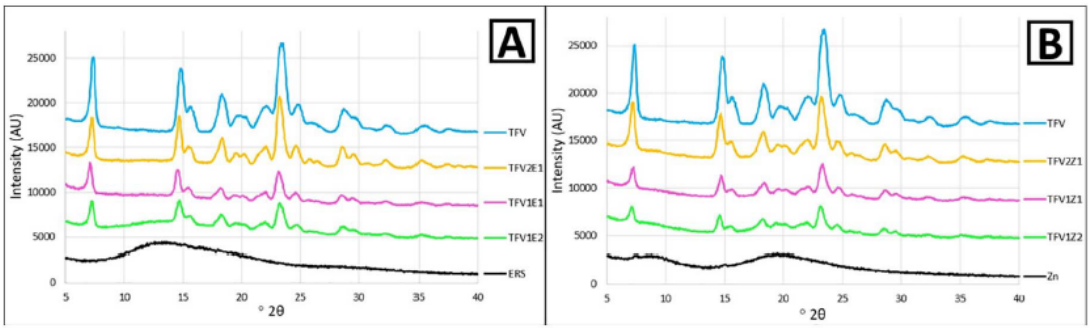

Fig. 1. X-ray diffraction patterns of pure TFV, ERS and the granules combining them (A); and TFV, Zein and the granules combining them (B).

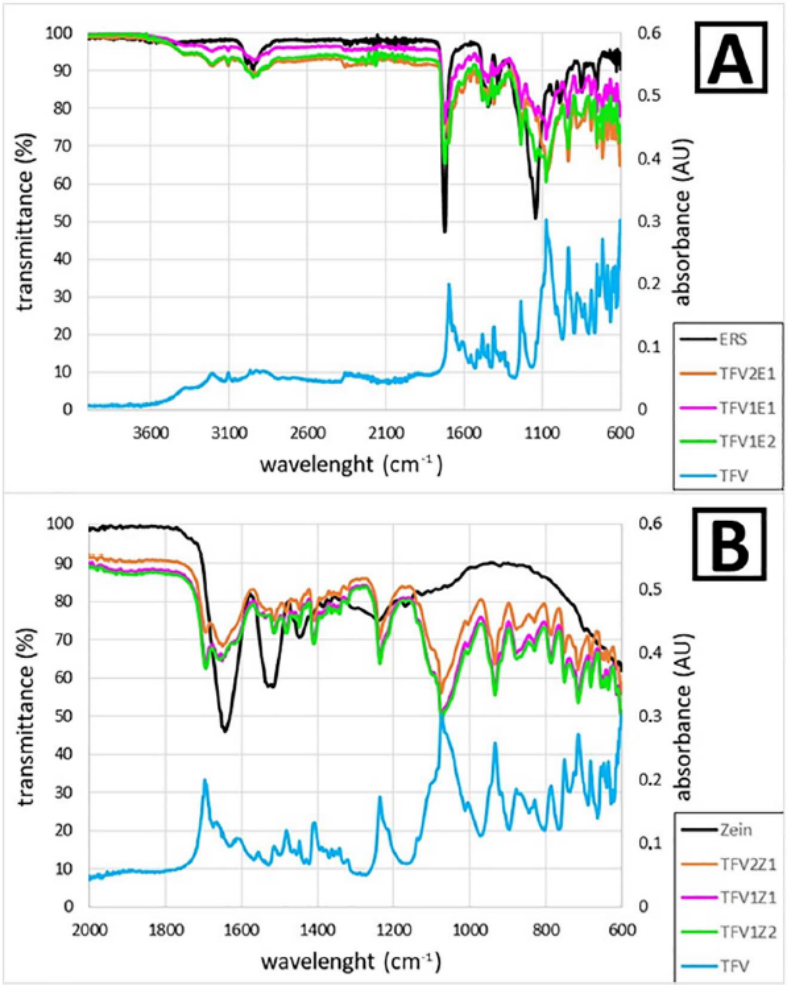

Fig. 2. FTIR-ATR spectra of TFV, ERS and their combinations (A) and TFV, Zein and their combinations (B). The absorbance spectra (right axes) correspond to TFV.

Zein also has two bands at around 1650 cm^{-1} and 1500 cm^{-1} due to its amide groups (Brahatheeswaran et al., 2012), which are particularly notable in the granules with Zein and TFV. It has a broadband centred at around 3300 cm^{-1} corresponding to the amine group. This band is considerably smaller in the granules, which suggests a possible hydrogen bonding between the drug and the polymer (Huang et al., 2013). However, the fingerprint region of the granules is exactly equal to pure TFV, so this interaction can be discarded.

This analysis therefore confirms that there is no interaction between the drug and the polymers during the preparation of the granules. This is important because it ensures that the TFV remains unchanged and retains its therapeutic potential.

3.1.3. Thermal analysis

Fig. 3A and B show the TFV weight loss registered in TGA studies, where a slight loss is observed at around 90 °C (possibly due to loss of water as the TFV may have captured moisture during storage), and a higher degradation due to drug decomposition beginning at 315 °C, until a final loss of 35% of its weight.

ERS decomposes completely beginning at the same temperature as the decomposition of TFV, and the granules combining TFV and ERS have a weight loss proportional to their TFV/ERS ratio, indicating once again that there is no interaction between the granule components (Fig. 3A). Zein loses about 70% of its weight during the TGA test and decomposition begins at around 280 °C (Fig. 3B). Zein decomposition continues at higher temperatures, but requires a temperature of 800 °C for complete degradation (Brahatheeswaran et al., 2012). The weight loss in granules with TFV and Zein is consistent with their content in these substances as they have an intermediate behaviour between the pure materials.

The DSC tests reveal the physical status of the drug in the granules. The DSC curve of TFV shows an endothermic peak at 159.77 °C, an exothermic response at 222.25 °C, another sharp endothermic peak at 295.05 °C and a final decomposition after 315 °C (Fig. 4). Since the peak centred at 295 °C corresponds to TFV melting (National Center for Biotechnology Information), the thermal phenomena detected at 159 and 222 °C may be due to a crystalline transition. No clear peaks that may correspond to fusion or phase transition are observed in the DSC curve of ERS due to its amorphous nature, but a strong exothermic process begins at around 345 °C which may be associated to polymer degradation (Fig. 4A). All the characteristic peaks of the drug can be found at similar temperatures in the DSC curves for the granules with TFV and ERS. This again suggests that no interaction has occurred with ERS.

As with ERS, no peaks can be seen in the DSC curve of Zein (Fig. 4B). There is only a slight increase in the curve at around 129 °C, corresponding to the glass transition (Ghanbarzadeh and Oromiehi, 2008; Madeka and Kokini, 1996), and a slow degradation beginning at around 280 °C. All the characteristic peaks of the drug are present in the granules with TFV and Zein.

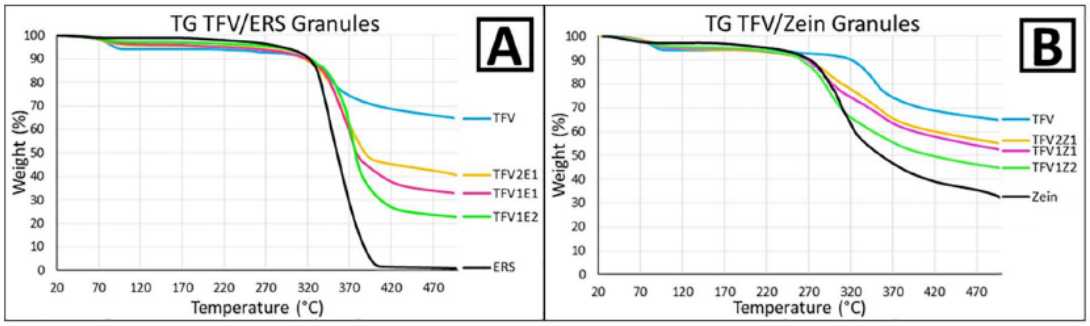

Fig. 3. TG curves corresponding to TFV, ERS and their combinations (A), and TFV, Zein and their combinations (B).

Finally, the thermal analysis of the granules was completed with HSM. The samples were visualized to correctly identify the changes detected with the other techniques and confirm there is no interaction between TFV and the polymers (Marras-Marquez et al., 2014).

The microscopic observation of TFV identifies it as a powdery crystalline substance, as the polarized light is able to pass through the sample. When the sample is heated, no change is observed until 167 °C, when some change in the crystallinity of the TFV can be surmised as the particles shrink slightly. The same occurs later at 220 °C, although in this case the crystal reorganization is more evident. This behaviour confirms the crystalline transformations at these temperature ranges, corresponding to the endothermic and exothermic peaks shown in the DSC curve. The sample finally melts at 264 °C.

Irregular dark (with polarized light) particles can be seen by visualizing the ERS, which confirms the amorphous character of the polymer. When the particles reach 56 °C they become brighter and the edges gradually deform, until at 130 °C they are all drops, so the characteristic softening of amorphous substances (glass transition) can clearly be said to be complete. It should be noted that when the sample is cooled, it hardens and maintains the shape of the droplets, another characteristic behaviour of amorphous substances. A similar behaviour is observed in the TFV/ERS granules regardless of the proportion of their components. Granules can be observed in all cases as irregularly-shaped particles that allow a little of the polarized light to pass. When the particles are heated, the glass transition of the ERS is observed to begin at 81 °C in the sample with most polymer (TFV1E2), although when the drug predominates (TFV2E1) or both substances are in equal parts (TFV1E1) this change cannot clearly be distinguished until 107–108 °C. In these cases, the actual glass transition temperature may be lower than observed, but the high TFV content, which remains unchanged, makes it difficult to visualize the changes in the edges of the particles. The next change occurs between 152 and 157 °C, where the edges of the particles are modified and some even shrink slightly, possibly due to the change in crystallinity of the TFV. Shortly after, from 160 °C, drops of ERS can be clearly visualized with TFV crystals inside. When the temperature rises to 212–222 °C, many more dots appear inside the drops, allowing the polarized light to pass through and indicating another change in the crystallinity of the TFV, which occurs at slightly lower temperatures when the granules contain more ERS, suggesting that the polymer in its rubbery state could help the TFV change its crystallinity. ERS burns at between 257 °C and 260 °C, giving the sample a dark colour, although the TFV crystals can still be observed until they melt at between 264 °C and 266 °C, and the crystals that allow the polarized light to pass can be seen to disappear.

In the thermomicroscope the Zein raw material is observed to consist of irregular (although some have a laminar form) amorphous particles, as the polarized light is unable to pass through them. The glass transition begins at 161 °C, a phenomenon most clearly observed in laminar particles whose edges are now less defined. This step to a rubbery state continues to occur progressively until 215 °C, when only drops of Zein can be seen (although with some small particles inside). Above 250 °C, these small particles also transition to a rubbery state and the Zein can be observed to darken gradually, until at 282 °C it is carbonized. TFV/Zein granules appear in all cases as irregular particles through which polarized light hardly passes. When the samples are heated they reveal a slight deformation at low temperatures (44–55 °C) in granules containing Zein in equal or greater quantities than TFV (TFV1Z1 and TFV1Z2). A more evident change occurs in these two types of granules at 170–174 °C, undoubtedly due to the glass transition of Zein. This change cannot be seen in the granules with twice as much TFV as Zein (TFV2Z1) due to the amount of drug they contain in proportion to the polymer. All the particles change completely between 200° and 214 °C, when they take on a rounded shape and display the TFV crystals more clearly, as the vitreous transition of the Zein has been completed and it is now in the form of drops. At 220–224 °C, as was the case of TFV/ERS granules, many more dots appear to pass through

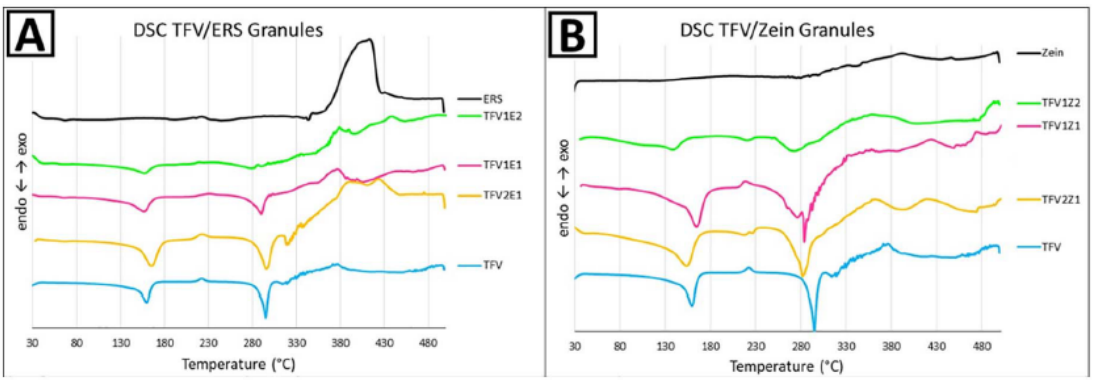

Fig. 4. DSC curves of TFV, ERS and their combinations (A) and TFV, Zein and their combinations (B).

polarized light, which is associated with the change in the crystalline state of TFV. At 255 °C the Zein darkens due to its decomposition, and the TFV crystals finally melt at 263–266 °C.

The analysis of the granules with the techniques described increases the knowledge of the characteristics and behaviour of the drug and the polymers used, but it specifically confirms that the TFV is not affected by the preparation process of the granules and guarantees that the drug will maintain its physicochemical and therapeutic properties in the final formulation.

3.2. Assessment of the compacts

In addition to characterizing the granules, the evaluation of the compacts –the final pharmaceutical dosage form– is of crucial importance. Their behaviour in SVF, mucoadhesive capacity and their behaviour following the release of TFV has therefore been evaluated. These tests allow us to determine the utility of the compact for clinical application in the prevention of HIV transmission.

3.2.1. Swelling behaviour

The swelling of the compacts is one of the factors controlling the drug release, as until the HPMC and CH matrix ceases to gel, the aqueous medium does not reach the granules and allow the diffusion of TFV. As can be seen in Fig. 5, all the formulations prepared undergo rapid swelling until reaching their maximum SR at 24–48 h, and the gel is finally eroded until it disappears completely.

The main difference observed when comparing these formulations with the HPMC/CH matrices that do not include the drug in granules (data obtained from Notario-Pérez et al. (2018) is the lower swelling attained by our compacts. This is due to the presence of ERS and Zein, both of which are incapable of forming a gel, which when interspersed between the HPMC and CH chains, reduces their ability to capture water and swell. In terms of the difference between the compacts containing Zein and ERS granules, it can be seen that the inclusion of Zein (TFV2Z1, TFV1Z1 and TFV1Z2) causes the gel to capture even less water than with ERS. This difference is much more noticeable when the proportion of the hydrophilic polymer is equal to (TFV1Z1) or lower than (TFV2Z1) TFV.

The maximum SR in the standard formulations (HPMC/CH) is achieved in all cases at 72 h, whereas –as already mentioned– in our formulations it is reached at 24–48 h. The degree of swelling is invariably greater when the polymer matrix contains more HPMC, since the formulations in Fig. 5B always swell more than their equivalents in Fig. 5A, and these in turn swell more than the batches in Fig. 5C. It should be noted that a period is observed in some formulations where the SR remains constant after reaching the maximum degree of swelling. This period becomes longer as the proportion of CH increases, as the gel it forms is more difficult to erode, whereas the inclusion of HPMC causes the gel to be easily erodible in the SVF.

The results of the swelling test indicate that the formulations with a lower maximum SR and more sustained swelling (C3 batches) will be the most acceptable to users due to their improved comfort.

3.2.2. Release study

As shown in Fig. 6, all formulations prepared with ERS or Zein allow the sustained release of TFV. The incorporation of TFV-loaded granules can be observed to reduce the rapid release of the drug occurring with the standard formulations in the first 6 h from 25 to 30% to 15–20%. This is because the drug must not only dissolve in the medium and diffuse through the gel, but must also diffuse through the Zein or ERS that accompanies the drug.

Although the TFV release profile is very similar in all the formulations, those containing ERS are able to hold the drug slightly longer than those containing Zein. Of all the formulations containing ERS, those containing TFV2E1 granules have a more sustained release profile, which is to be expected since the drug must diffuse through a thicker ERS layer. This reduction in the initial drug release rate also determines the length of time taken to completely release the TFV. Thus, compared to the 72 h for the standard formulation, 96 h of drug release is achieved in most formulations containing TFV granules, and even up to 144 h when the granulate in the compacts has twice as much ERS as TFV.

Finally, it is worth noting that there are minimal differences between the graphs in Fig. 6A, B and C, with very similar profiles regardless of the proportion of HPMC and CH in the batches. This confirms that the HPMC/CH combination forms a very robust mixed gel that is capable of maintaining its properties despite changes in the proportion of the two polymers (Notario-Pérez et al., 2018).

The granulation of TFV with ERS or Zein therefore prolongs the controlled release time of TFV, and allows an optimal formulation to be obtained.

TFV release profiles obtained in the drug release studies were adjusted to different mathematical model-dependent methods (zero order, first order, Higuchi, Hixson-Crowell, Hopfenberg, Korsmeyer-Peppas and Weibull).

As can be seen in Table 4, all batches have a good fit to the kinetics of Higuchi, Hixson-Crowell, Hopfenberg, Korsmeyer-Peppas and Weibull. Batches with the polymer mixtures C1 and C2 (those with an equal or greater proportion of HPMC to CH) are observed to have a better fit to the Hixson-Crowell model, whereas the batches including C3 (where CH predominates) fit the Weibull kinetic better.

According to the literature, the fact that the compacts fit the Hixson-Crowell kinetic indicates that the drug release from the compacts occurs through drug dissolution in planes parallel to the surface, so the compact decreases proportionally in size over time and the geometry remains constant. In our formulations, the most likely occurrence is that the outer layers of the HPMC/CH mixture gel and swell proportionally, maintaining their shape constant for the first 24–48 h. During this time, the drug-loaded ERS/Zein granules in the outer layers are able to release the drug as the hydrophilic polymers gel, always proportionally over time. After 48 h, the gel begins to erode (as seen in the swelling tests, Fig. 5) –also progressively– so the TFV-loaded granules in the inner layers of the compact are exposed to the medium and can also release the drug. This explains why the batches with mixtures C1 and C2 have the best fit to this mathematical model, since it is where both swelling and erosion take place continuously and progressively, while batches C3 (with more CH) have a period in which the size remains constant once the maximum SR has been reached.

As already mentioned, the C3 batches are better fitted to the Weibull kinetics. This model is generally applied to explain the release of the drug from matrix tablets, and in our case it has a better fit with batches containing more CH as gelling is much faster and the gel is more difficult to erode, so it remains practically constant until all the drug has been released.

When comparing the drug release constants obtained with the different models (shown in Table 5), it can be seen that very similar results are obtained in the kinetics of Higuchi, Hixson-Crowell, Hopfenberg and Weibull, where all the batches containing the TFV1E2 granules (with twice as much ERS as TFV) have the lowest values for the drug release constant, so the TFV release occurs in a more sustained way. In all cases the more HPMC the batch contains the lower the value of the constant (C2 < C1 < C3), offering more effective control over drug release.

In the fit to the Korsmeyer-Peppas model, the value of n is also obtained in addition to the constant (K_{KP}), indicating the drug release mechanism. Most of the batches have values of $n = 0.89$, indicating that the release follows Case II, or $n > 0.89$, corresponding to a Supercase II type release. Both Case II and Supercase II involve a structural modification of the polymer matrix, corresponding here to the gelling of the compacts and their subsequent erosion. However, there are also some batches with n values between 0.45 and 0.89, which according to the literature implies an abnormal transport of the drug,

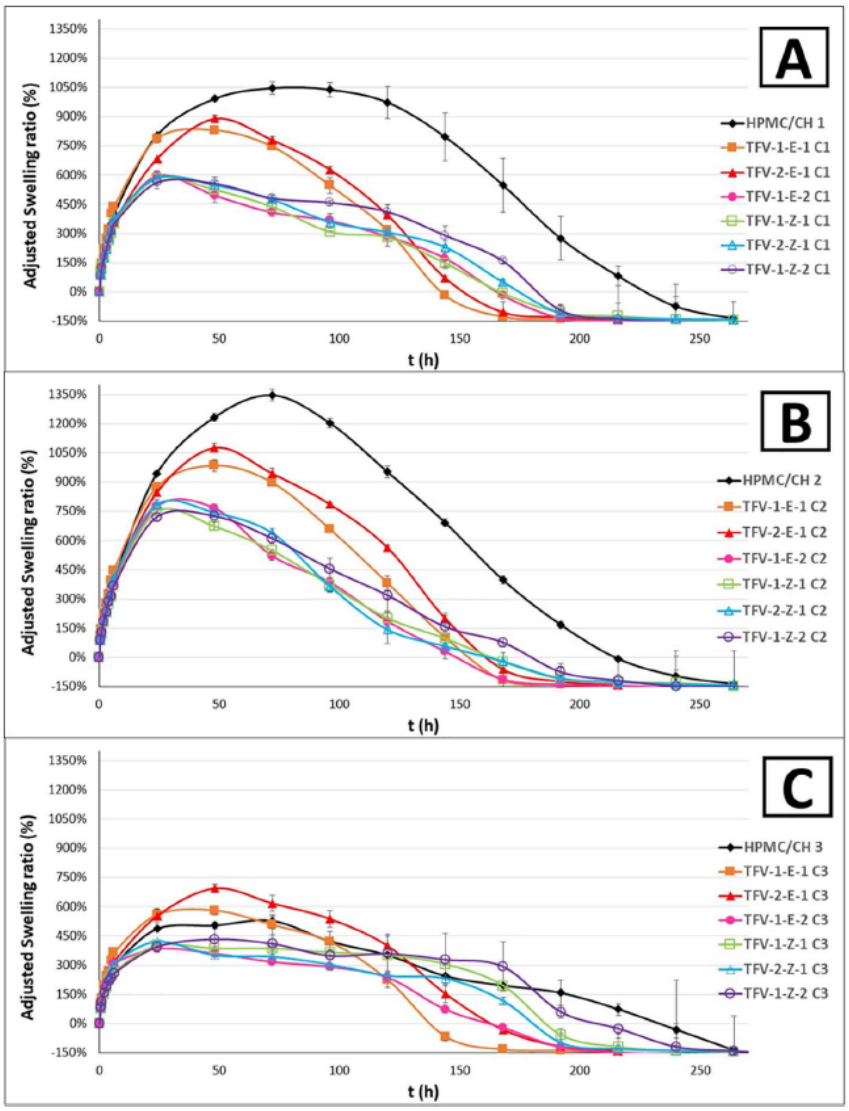

Fig. 5. Adjusted swelling ratio profiles obtained from batches including drug-loaded granules in HPMC/CH matrices with equal ratios of both polymers (A), more HPMC than CH (B) and more CH than HPMC (C). All of them are compared with standard formulations of HPMC/CH (Notario-Pérez et al., 2018).

where diffusion and matrix modification occur simultaneously. In these batches the release of the drug is affected by both the gelling and erosion of the polymers, coupled with the ability of ERS and Zein to control drug diffusion (although values of n are always close to 0.89, indicating that structural modification is the predominant release control mechanism). Finally, it should also be noted that the K_{KP} values for the batches containing the ERS granules are lower than for the Zein batches, indicating that the ERS granules are better able to modulate the release of the TFV.

3.2.3. Mucoadhesion test

The mucoadhesion of the formulation to the vaginal mucosa is a crucial factor for its clinical efficacy (Caramella et al., 2015). The optimal formulation should be able to remain adhered for long enough for the complete release of TFV, but it is also important that once the drug has diffused, the unloaded gel does not remain for an excessive time in the vagina, as this would cause discomfort to the user and could make it difficult to administer the next dose (Notario-Pérez et al., 2017).

To help compare and interpret the results, Fig. 7 shows the mucoadhesion of each batch and the time taken to release the TFV. As can be seen, the standard HPMC and CH formulations had a 96-h bioadhesion time to the mucosa, the same time it took to release all the drug (Notario-Pérez et al., 2018). This could pose a problem, since to achieve an effective formulation that allows administration over a longer period, it is not sufficient simply to lengthen the TFV release time; its mucoadhesive capacity must also be improved.

As can be seen in Fig. 7, the mucoadhesion time markedly improves in all batches prepared with the granules. The reasons are twofold: the TFV is granulated with ERS and Zein, two polymers with proven adhesive capacity (Alqahtani et al., 2017; Notario-Pérez et al., 2017; Singh et al., 2015); and, as seen in the swelling tests (Fig. 5), the inclusion of the granules causes the compact to capture less water and therefore form a denser gel. The result is that a larger area of the polymer is in contact with the vaginal mucosa and can form bonds with it; and the lower water gain in the gel means the bonds do not need to support so much weight.

When batches include the TFV and Zein granules, the mucoadhesion time is set to 138–190 h, a significant improvement on the 96 h of the

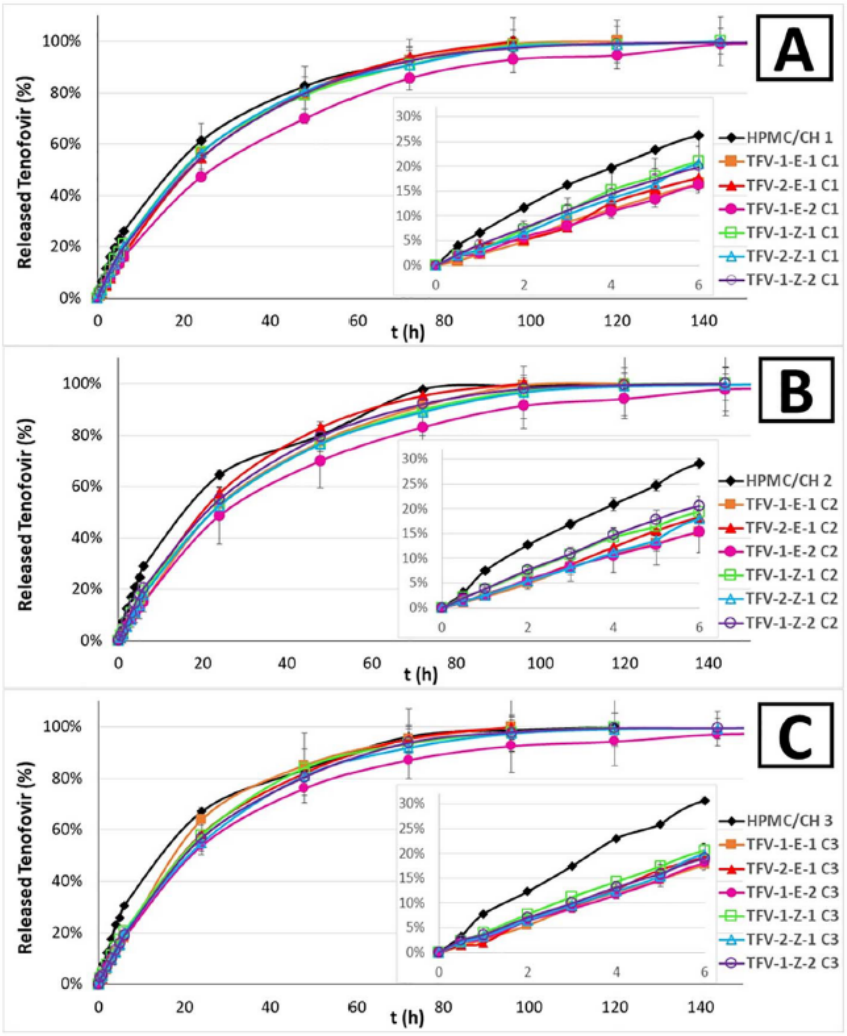

Fig. 6. TFV release profiles obtained from batches containing drug-loaded granules in HPMC/CH matrices with equal ratios of both polymers (A), more HPMC than CH (B) and more CH than HPMC (C). All of them are compared with standard formulations of HPMC/CH (Notario-Pérez et al., 2018).

Table 4
Correlation coefficients obtained when experimental data are fitted to the different mathematical models.

Batch	Correlation coefficients (r^2)						
	Zero order	First order	Higuchi	Hixson-Crowell	Hopfenberg	Korsmeyer-Peppas	Weibull
TFV1E2 C1	0.9466	0.6990	**0.9904**	**0.9954**	**0.9872**	**0.9897**	**0.9981**
TFV1E2 C2	0.9399	0.6814	**0.9890**	**0.9916**	**0.9819**	**0.9940**	**0.9920**
TFV1E2 C3	0.9184	0.6775	**0.9835**	**0.9812**	**0.9679**	**0.9923**	**0.9981**
TFV1E1 C1	0.9242	0.6358	**0.9832**	**0.9978**	**0.9876**	**0.9895**	**0.9677**
TFV1E1 C2	0.9378	0.6663	**0.9862**	**0.9964**	**0.9943**	**0.9941**	**0.9308**
TFV1E1 C3	0.8990	0.6451	**0.9732**	**0.9907**	**0.9837**	**0.9985**	**0.9986**
TFV2E1 C1	0.9303	0.6935	**0.9856**	**0.9849**	**0.9957**	**0.9769**	**0.9936**
TFV2E1 C2	0.9184	0.6401	**0.9821**	**0.9918**	**0.9925**	**0.9903**	**0.9929**
TFV2E1 C3	0.9167	0.6248	**0.9833**	**0.9913**	**0.9923**	**0.9735**	**0.9903**
TFV1Z2 C1	0.9223	0.6768	**0.9874**	**0.9936**	**0.9807**	**0.9952**	**0.9962**
TFV1Z2 C2	0.9231	0.6588	**0.9886**	**0.9961**	**0.9841**	**0.9906**	**0.9886**
TFV1Z2 C3	0.9194	0.6945	**0.9842**	**0.9936**	**0.9801**	**0.9954**	**0.9946**
TFV1Z1 C1	0.9189	0.6435	**0.9879**	**0.9953**	**0.9841**	**0.9858**	**0.9746**
TFV1Z1 C2	0.9326	0.6784	**0.9908**	**0.9969**	**0.9871**	**0.9933**	**0.9883**
TFV1Z1 C3	0.9083	0.6709	**0.9817**	**0.9888**	**0.9724**	**0.9962**	**0.9981**
TFV2Z1 C1	0.9168	0.6609	**0.9849**	**0.9935**	**0.9803**	**0.9920**	**0.9856**
TFV2Z1 C2	0.9315	0.6623	**0.9866**	**0.9955**	**0.9847**	**0.9934**	**0.9925**
TFV2Z1 C3	0.9204	0.6768	**0.9843**	**0.9930**	**0.9793**	**0.9945**	**0.9958**

Bold values correspond with the kinetics with the best fit.

Table 5
TFV release kinetics from the different batches showing the kinetic constants for the models with the best fit.

Batch	Higuchi	Hixson-Crowell	Hopfenberg	Korsmeyer-Peppas		Weibull
	K_H	K_{HC}	K_{HF}	K_{KP}	n	K_w
TFV1E2 C1	0.105	0.0062	0.0079	0.031	0.86	0.026
TFV1E2 C2	0.103	0.0059	0.0076	0.028	0.91	0.024
TFV1E2 C3	0.107	0.0063	0.0080	0.033	0.88	0.027
TFV1E1 C1	0.115	0.0080	0.0096	0.023	1.05	0.042
TFV1E1 C2	0.114	0.0081	0.0096	0.024	0.99	0.045
TFV1E1 C3	0.119	0.0097	0.0106	0.027	1.05	0.041
TFV2E1 C1	0.115	0.0095	0.0103	0.035	0.85	0.036
TFV2E1 C2	0.117	0.0097	0.0105	0.027	1.00	0.040
TFV2E1 C3	0.117	0.0097	0.0104	0.028	1.01	0.040
TFV1Z2 C1	0.119	0.0074	0.0090	0.042	0.82	0.036
TFV1Z2 C2	0.112	0.0075	0.0091	0.040	0.86	0.037
TFV1Z2 C3	0.114	0.0078	0.0093	0.040	0.82	0.039
TFV1Z1 C1	0.112	0.0076	0.0091	0.038	0.89	0.038
TFV1Z1 C2	0.109	0.0071	0.0088	0.040	0.83	0.034
TFV1Z1 C3	0.114	0.0078	0.0093	0.042	0.84	0.038
TFV2Z1 C1	0.112	0.0075	0.0091	0.036	0.89	0.037
TFV2Z1 C2	0.111	0.0070	0.0087	0.027	0.96	0.033
TFV2Z1 C3	0.113	0.0075	0.0091	0.036	0.87	0.036

standard formulation (Fig. 7). Although not always the case, the mucoadhesion can be assumed to be greater in the batches that include more Zein, where the complete release of the TFV always takes place at 120 h, so the loss of the formulation never occurs when there is still drug remaining inside. There is a much clearer effect on the formulation when the compacts include TFV granules obtained using ERS. A marked improvement in mucoadhesion is again observed in all cases, with up to 140–238 h of bioadhesion (Fig. 7). It is also clear that adhesion improves the less ERS there is in the granules, and that the C1 batches (combining HPMC and CH in equal parts) remain attached to the vaginal mucosa for longest, followed by C2 (more HPMC than CH) and finally batches C3 (more CH than HPMC), which adhere for the least time. The batches with the TFV1E2 granules are particularly significant as they show the greatest improvement, and extend the TFV release time to 144 h. The mucoadhesion time in these three batches is between 150 and 165 h, always longer than the drug release time, but not remaining beyond this time, unquestionably making them the choice formulations for clinical application. In our previous studies, we achieved mucoadhesive tablets that could release Tenofovir during 72 h (Pereira et al., 2014), but the tablets developed in the present work are able to double the time during which the drug is released. This is possible thanks to a double mechanism that shows the novelty of this work, combining the well-known swellable polymers with a drug granulation, resulting into a controlled release system that never has been explored. Moreover, we find some examples of vaginal tablets in the literature that highlight the importance of the formulation developed in the present work. For example, we found an example of multilayer tablets that include other antivirals such as Acyclovir or Dapivirine, which are only able to control the release for 8 h (McConville et al., 2016). The same applies to vaginal tablets that include Tenofovir, the drug that we use in our research, that require daily application, as in the case of the tablets developed by McConville et al. (2013) or by Pereira et al. (2014).

But this work not only stands out for its novelty, but also for the advantages that the application of this system would imply, since it has been proven that low adherence is linked with frequent dosing, so a formulation like this one, that will notably decrease dosing, probably would increase adherence and efficacy of prophylactic treatment.

However, it is necessary that the *in vitro* studies could test the key indicators of the formulation in an *in vivo* context, although for that it is may not necessary to imitate closely the *in vivo* environment (Tuğcu-Demiröz et al., 2013). Thus, we have used an alternative method to the classic Franz cell system. In our method, we use as lower volume of aqueous fluid –which is more representative of the vaginal volume-, and this medium is simulated vaginal fluid, in order to imitate the conditions of pH and osmolality of an *in vitro* test. The speed (15 rpm) and the movement (in and oscillating bath) are also more representative of the reality. Anatomic and physiologic parameters also must be considered. As an example, vaginal clearance could suppose a problem in maintaining protective Tenofovir concentrations in vaginal fluids, but mucoadhesive formulations have demonstrated to be an option to resist both gravity and clearance effects (Campaña-Seoane et al., 2014). In addition, our test -due to the lower volume of medium and the higher volume replaced in every sample- simulates this clearance better than any other (every day 15 mL from a total of 80 mL are replaced by new simulated vaginal fluid, which suppose a daily "clearance" of 18.75% of the medium). It is also worth mention that in our test, the inhibitory concentration 50 (IC50) of TFV –found between 1.08 and 1.22 μM (Musumeci et al., 2017), is already reached in the first taken sample (quantified at 30 min after tablet administration), and a concentration over this value is achieved throughout the trial despite the aforementioned speed of renewal of the medium, so we think that similar results will be obtained in *in vivo* tests. In spite of that, there are obviously still differences, and an *in vitro* test is always a preliminary

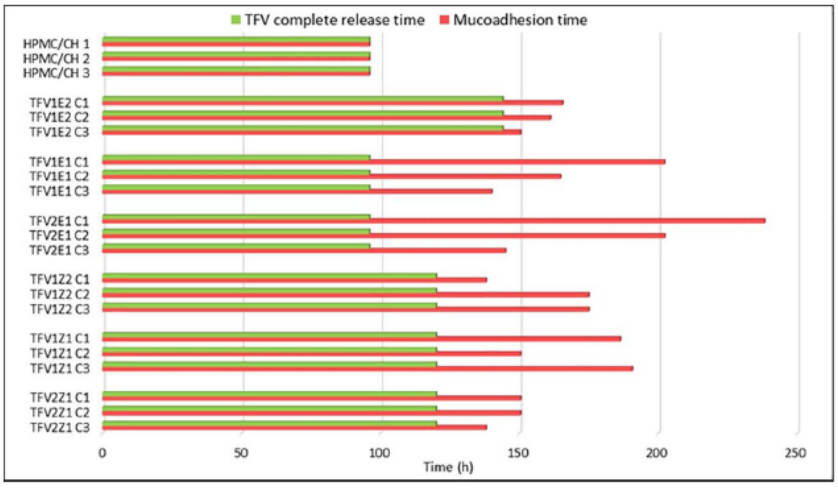

Fig. 7. Time of permanence adhered to the vaginal mucosa of the different batches, grouped with the duration of complete TFV release from each batch. Standard formulations of HPMC/CH (Notario-Pérez et al., 2018) are also shown.

trial that must be confirmed in subsequent *in vivo* studies.

On the other hand, regarding mucoadhesion in an *in vivo* context, the results obtained are probably closely to the real ones, since despite the vaginal fluid clearance could accelerate erosion, the constant pressure exerted by the vagina -which is an elastic muscular duct that tends to close on itself- will suppose that the tablet would be more protected and it will be more difficult to be detached.

Therefore, the excellent results showed in *in vitro* test make us be hopeful about the results that this formulation will achieve in future *in vivo* studies.

4. Conclusions

Thermal analysis, IR spectrophotometry and X-ray diffraction techniques have proven that TFV does not alter during the granulation process of TFV with ERS or Zein.

The drug-loaded granules with ERS or Zein significantly decrease the initial rapid release of TFV and cause a lower swelling of the compacts, making them more comfortable and lengthening the time they remain attached to the vaginal mucosa.

The incorporation of TFV1E2 granules in compacts containing HPMC/CH 1:1.9 allows the optimal formulation to be developed for the prevention of sexual transmission of HIV, since they are capable of releasing the drug in a sustained manner for 144 h, remain attached to the vaginal mucosa for 150 h, and have moderate swelling (as they contain less HPMC), which makes them more comfortable for women.

Abbreviations

ACDP	Anhydrous calcium hydrogen phosphate
ASR	Adjusted swelling ratio
AU	Arbitrary units
CH	Chitosan
DSC	Differential scanning calorimetry
ERS	Eudragit® RS
FTIR-ATR	Fourier transform infrared attenuated total reflection spectroscopy
HIV	Human immunodeficiency virus
HPMC	Hydroxypropylmethyl cellulose
HSM	Hot stage microscopy
MgSt	Magnesium stearate
PVP	Polyvinylpyrrolidone
SR	Swelling ratio
SVF	Simulated vaginal fluid
TFV	Tenofovir
TGA	Thermogravimetric analysis

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Author contributions

Fernando Notario-Pérez, Roberto Ruiz-Caro and María-Dolores Veiga designed and planned the experiments. Fernando Notario-Pérez, Araceli Martín-Illana, Raúl Cazorla-Luna and Juan Peña conducted the experiments. All authors have approved the final article. María-Dolores Veiga is the senior author and project leader.

Conflicts of interest

The authors declare no conflict of interest.

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CAPÍTULO V

TENOFOVIR HOT-MELT GRANULATION USING GELUCIRE® TO DEVELOP SUSTAINED-RELEASE VAGINAL SYSTEMS FOR WEEKLY PROTECTION AGAINST SEXUAL TRANSMISSION OF HIV

pharmaceutics

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Article

Tenofovir Hot-Melt Granulation using Gelucire® to Develop Sustained-Release Vaginal Systems for Weekly Protection against Sexual Transmission of HIV

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Abstract: Hot-melt granulation is a technique used to obtain granules by dispersing a drug in polymers at a high temperature. Tenofovir, an antiretroviral drug with proven activity as a vaginal microbicide, was dispersed in melted Gelucire® (or a mixture of different Gelucire®) to obtain drug-loaded granules. Studies performed on the granules proved that the drug is not altered in the hot-melt granulation process. The granules obtained were included in a matrix formed by the hydrophilic polymers hydroxypropylmethylcellulose and chitosan to obtain vaginal tablets that combine different mechanisms of controlled release: The Gelucire® needs to soften to allow the release of the Tenofovir, and the hydrophilic polymers must form a gel so the drug can diffuse through it. The studies performed with the tablets were swelling behavior, Tenofovir release, and ex vivo mucoadhesion. The tablets containing granules obtained with Tenofovir and Gelucire® 43/01 in a ratio of 1:2 in a matrix formed by hydroxypropylmethylcellulose and chitosan in a ratio of 1.9:1 were selected as the optimal formulation, since they release Tenofovir in a sustained manner over 216h and remain attached to the vaginal mucosa throughout. A weekly administration of these tablets would therefore offer women protection against the sexual transmission of HIV.

Keywords: controlled release; ex vivo bioadhesion; Gelucire®; Human Immunodeficiency Virus; mucoadhesive vaginal compacts; Tenofovir

1. Introduction

The hot-melt granulation technique consists of dissolving or dispersing an active principle in one or more melted polymers in order, after cooling, to obtain granules with a defined structure [1]. The melt entropy of the different components determines whether they will crystallize simultaneously, in turn conditioning the microstructure of the granules in the solid state [2].

The most common applications of this technique in pharmaceutical development are targeted drug release, masking the taste of active substances, and particularly improving the solubility of poorly soluble drugs [3]. In the latter case, solid dispersions of the drug are usually prepared in an inert hydrophilic matrix, causing the active principle to convert from its crystalline state to an amorphous form and improving its solubility [4]. However, this technique could also be applied in reverse to delay the dissolution of water-soluble drugs, which would aid the development of sustained-release systems

capable of releasing the drug at a given rate, thereby achieving a constant concentration of the active principle at the site of action [3,4]. Some references to this possibility can be found in the literature: Mini-matrices have been developed where the drug is found in polymers as diverse as ethyl cellulose and xanthan gum [5], polyvinylpyrrolidone (PVP) [6], thermoplastic polyurethane [7] or ethylene vinyl acetate and polyethylene oxide [8]. One alternative yet to be explored would be to develop these systems using as carriers various Gelucire®, a group of amphiphilic excipients commonly used to develop sustained-release matrices [9,10]. Another advantage is that Gelucire® is commercialized with different melting points and hydrophilic-lipophilic balances (HLB), offering a wide range of options from which to select the most suitable for our purposes [11].

This last option—developing systems for the sustained release of drugs—raises the possibility of applying this technique to produce a vaginal microbicide to prevent the sexual transmission of the human immunodeficiency virus (HIV). A vaginal microbicide is any suitably formulated compound which, when applied in the vagina before intercourse, has the ability to prevent or reduce the transmission of sexually-transmitted diseases. The latest published data reflect the gender inequality in HIV protection, with an estimated 18.6 million women and girls living with the virus, and three young women infected every four minutes [12]. This explains the growing interest in recent decades in developing this type of formulations as a tool to protect women from the sexual acquisition of HIV.

Tenofovir (TFV) is an antiretroviral drug, specifically a nucleotide analogue reverse transcriptase inhibitor, approved by the Food and Drug Administration and recommended by the World Health Organization for its use as a first-line drug for the treatment of HIV [13]. It has also been extensively studied for the development of vaginal microbicides, mainly since the publication in 2010 of the results of the CAPRISA 004 trial, in which a 1% TFV gel showed a 39% effectiveness in protecting against HIV infection [14]. However, the fact that it required a daily application reduced the effectiveness of the formulation, so the current trend is to develop sustained-release formulations that require less frequent administration [15]. TFV is a hydrophilic drug that is soluble in aqueous media, such as vaginal fluid, requiring techniques to obtain sustained-release formulations that slow its dissolution rate in the vaginal environment.

The aim of this work is to prepare granules of TFV by hot-melt granulation using one or more Gelucire® containing mono-, di- and triglyceride esters of fatty acids (C8 to C18), –Gelucire® 39/01 (G39) and Gelucire® 43/01 (G43)–, which are characterized by their low HLB value (HLB = 1) and low melting point (39 °C and 43 °C respectively). However, rather than manufacturing matrices, the granules are incorporated in a hydrophilic matrix formed by a combination of hydroxypropylmethyl cellulose (HPMC) and chitosan (CH), two polymers that are capable of swelling in the presence of vaginal fluid and have proven to be an excellent choice for the development of sustained drug-delivery systems [16]. Vaginally administered tablets could then be developed in which the release of the drug would be conditioned by multiple factors. A Gelucire® with a predominantly lipophilic rather than hydrophilic character would prevent the dissolution of the granules in the vaginal medium, while their low melting point would cause Gelucire® to soften at body temperature and allow the vaginal fluid to diffuse through it and dissolve the drug; alternatively, the combination of HPMC and CH would swell with the vaginal fluid and form a gel which would initially delay the arrival of the vaginal fluid at the granules, and subsequently hinder the diffusion of the dissolved drug through the gel to reach the vaginal environment. The aim is to develop mucoadhesive systems that offer weekly protection against HIV infection and encourage greater adherence to the use of the microbicide, due to their lower frequency of administration, and consequently achieve greater protective efficacy.

2. Materials and Methods

2.1. Materials

Gelucire® 39/01 (G39, lot: 3E3701-2) and Gelurice® 43/01 (G43, lot: 1E5203-2) were a gift from Gattefossé (Saint-Priest, France). Tenofovir (TFV, lot: FT104801401) was supplied by Carbosynth

Limited (Berkshire, UK). Chitosan, whose properties were characterized experimentally determining that it has a molecular weight of 32.1 kDa—being therefore a low-molecular-weight chitosan-, with an intrinsic viscosity of 24.75 dL/g and a degree of deacetylation of 54. 7% [17] (CH, lot: 0055790), was provided by Guinama (La Pobla de Vallbona, Spain). Hydroxypropylmethylcellulose—Methocel® K 100 M (HPMC; lot: SB13012N31) was kindly supplied by Colorcon Ltd. (Kent, UK). Anhydrous calcium hydrogen phosphate—Emprove®—(ACDP; lot: K93487944416) was supplied by Merck (Darmstadt, Germany). Polyvinylpyrrolidone—Kollidon® 30—(PVP; lot: 98-0820) was purchased from BASF Aktiengesellschaft (Ludwigshafen, Germany). Magnesium stearate PRS-CODEX (MgSt; lot: 85269 ALP) was acquired from Panreac (Barcelona, Spain).

All other reagents in this study were of analytical grade and used without further purification. Demineralized water was used in all cases.

2.2. Preparation of the Granules

The granules were prepared using G39, G43 and a mixture of both in equal proportions in the carrier/TFV ratios, shown in Table 1. The granules were obtained by first melting the Gelucire® in an oil bath (Selecta® Univeba-401, Barcelona, Spain), taking care to ensure the temperature never exceeded 50°C. Once completely melted, the TFV was incorporated and the mixture was stirred until the drug was homogeneously dispersed. The mixture was then spread over a smooth non-stick surface and left to stand at room temperature for a few minutes to solidify. Finally, the solidified mixture was forced through a 1 mm mesh to obtain the granules. Thus, this ensured that all granules obtained had a size of < 1mm in diameter.

Table 1. Granule composition (mg).

Granules	Tenofovir	Gelucire® 39	Gelucire® 43
TFV-1-G43-2	30		60
TFV-1-G43-1	30		30
TFV-2-G43-1	30		15
TFV-1-G41-2	30	30	30
TFV-1-G39-2	30	60	

2.3. Characterization of the Granules

2.3.1. Infrared Spectroscopy

Fourier Transform Infrared Attenuated Total Reflection Spectroscopy (FTIR-ATR) was used to characterize the pure materials and prepared granules with a Perkin-Elmer spectrophotometer equipped with a MIRacle™ accessory designed for FTIR-ATR measurements (Perkin-Elmer, Waltham, MA, USA).

2.3.2. Thermal Analysis

Hot-stage microscopy studies were performed between 30 °C and 350 °C. Approximately 1 mg of each sample was placed on a microscope slide with a cover on a Kofler stage and heated at a rate of 2 °C/min. A thermogalen microscope fitted with the Kofler stage (Leica, Wetzlar, Germany) was used for the microscopy examinations.

Thermogravimetric analysis (TGA) and differential scanning calorimetry (DSC) were done in an SDT-Q 600 TA Instruments TG/DTA analyzer (TA Instruments, New Castle, DE, USA). In this case, about 5−10 mg of samples were placed in a pinholed aluminium sample pan with a lid and heated in atmospheric air to between 30 °C and 500 °C at a rate of 10 °C/min.

2.3.3. X-ray Diffraction

An automated Philips X'Pert-MPD X-ray diffractometer with Bragg–Brentano geometry (Malvern Panalytical Ltd., Royston, UK) was used to obtain the X-ray diffraction patterns of pure materials and all granulated systems. Monochromatized Cu-Kα radiation (λ = 1.5406 Å) was irradiated over the samples at 45 kV, 40 mA and a time per step of 2 s, and analyzed between 2θ angles of 5° and 50°.

An X-ray thermodiffraction of the TFV was also performed using an X'Pert PRO MPD in theta-theta configuration with an Anton Paar HTK1200 high-temperature camera (Malvern Panalytical Ltd., Royston, UK). Monochromatized Cu-Kα radiation (λ = 1.5406 Å) was irradiated over the sample at 45 kV and 40 mA. The sample was heated to 5 °C/min and analyzed between 2θ angles of 5° and 50° at 25 °C, 150 °C, 175 °C and 225 °C. The sample was then cooled at the same rate and analyzed at 200 °C, 150 °C and 25 °C under the same conditions.

2.4. Preparation of the Tablets

Vaginal tablets were developed with a combination of hydrophilic polymers (HPMC and CH) containing the granules developed previously. Each granulated system was in turn included in three different combinations of HPMC and CH. HPMC, CH and ACDP were mixed, and the mixture was then wetted with a solution of PVP in ethanol to produce a mass, which was granulated using a 1 mm mesh. Three granulates were obtained (C1, with a ratio of 1:1 HPMC/CH; C2, with a ratio of 1.9:1 HPMC/CH; and C3, with a ratio of 1:1.9 HPMC/CH) and dried at room temperature for 24 h. Lastly, each granulate was mixed with the TFV granules for a final content of 30 mg of drug and 290 mg of hydrophilic polymer per tablet. All granulates were prepared by forcing them through the same mesh to ensure that all granules—polymeric and TFV-loaded granules—had the same size (<1 mm in diameter), and the blends were homogeneous. MgSt was added to the total dried granules before tableting. The final content of each batch is shown in Table 2.

Table 2. Tablet composition (mg/unit).

Batch	HPMC	CH	ACDP	PVP	TFV	G43	G39	MgSt
TFV-1-G43-2 C1	145	145	45	27	30	60		3
TFV-1-G43-2 C2	190	100	45	27	30	60		3
TFV-1-G43-2 C3	100	190	45	27	30	60		3
TFV-1-G43-1 C1	145	145	45	27	30	30		3
TFV-1-G43-1 C2	190	100	45	27	30	30		3
TFV-1-G43-1 C3	100	190	45	27	30	30		3
TFV-2-G43-1 C1	145	145	45	27	30	15		3
TFV-2-G43-1 C2	190	100	45	27	30	15		3
TFV-2-G43-1 C3	100	190	45	27	30	15		3
TFV-1-G41-2 C1	145	145	45	27	30	30	30	3
TFV-1-G41-2 C2	190	100	45	27	30	30	30	3
TFV-1-G41-2 C3	100	190	45	27	30	30	30	3
TFV-1-G39-2 C1	145	145	45	27	30		60	3
TFV-1-G39-2 C2	190	100	45	27	30		60	3
TFV-1-G39-2 C3	100	190	45	27	30		60	3

HPMC: Hydroxypropylmethylcellulose; CH: Chitosan; ACDP: Anhydrous calcium hydrogen phosphate.

The tablets were produced using a press similar to the one used for preparing solid samples for analysis by IR spectroscopy. A constant pressure of 5 tons was applied for four minutes using a punch. The resulting tablets were cylindrical in shape with a diameter of 13 mm and a height of 2.5–2.8 mm.

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2.5. Assessment of the Tablets

2.5.1. Swelling Behavior

The method described by Ruiz-Caro et al. [18] was used to analyze the swelling behavior of each batch in simulated vaginal fluid (SVF) [19]. The test was done in triplicate in a shaking water bath (Selecta® UNITRONIC320 OR, Barcelona, Spain) at 37 °C and 15 opm. The discs were removed from the medium at predetermined times, placed on filter paper to eliminate excess liquid and weighed. The swelling ratio (SR) was calculated following Equation (1) where T_s and T_d correspond to the swollen and dry tablet weights respectively:

$$SR = \frac{T_s - T_d}{T_d} \cdot 100. \quad (1)$$

Since the initial tablet weight varies depending on the amount of Gelucire® it contains, the SR of different batches was compared by determining the adjusted swelling ratio (ASR), where the weight increment is related to the amount of swellable polymer, as shown in Equation (2) where SR, T_d and SP correspond respectively to the swelling ratio determined by Equation (1), the dry tablet weight, and the amount of swellable polymer in the tablet:

$$ASR = \frac{SR \cdot T_d}{SP}. \quad (2)$$

2.5.2. Release Study

The release of TFV from the batches was evaluated with the method described by Sánchez-Sánchez et al. [20]. The tablets were immersed in 80 mL of SVF in a borosilicate glass bottle and placed in a shaking water bath (Selecta® UNITRONIC320 OR, Barcelona, Spain) at 37 °C and 15 opm. The test was performed in triplicate. Release tests are performed in sink conditions because the solubility of TFV in SVF, measured at room temperature, is 4 mg/mL. 5 mL samples were taken and filtered at given times, and the medium was replaced with the same volume of SVF at the same temperature. The TFV released was quantified by UV spectroscopy at a wavelength of 260 nm in a Shimadzu® UV-1700 (Kyoto, Japan).

The similarity factor (f_2) (Equation (3)) was calculated in order to statistically demonstrate the differences between the batches.

$$f_2 = 50 \times \log \left\{ \left[1 + \left(\frac{1}{n} \right) \sum_{j=1}^{n} W_j |R_j - T_j|^2 \right]^{-0.5} \times 100 \right\}. \quad (3)$$

This is a model-independent index described by Moore and Flanner [21], where n is the number of samples for each dissolution test, R_j and T_j are the drug release percentage at each time for the reference and test product respectively, and W_j is a weight factor, which in this study is equal to 1. A value of $f_2 > 65$ means similarity between the profiles of over 95%, while $f_2 < 65$ indicates that the profiles are significantly different [22].

Finally, the experimental drug-release data were fitted to different model-dependent methods (zero order, first order, Higuchi, Hixson-Crowell, Hopfenberg and Korsmeyer-Peppas models) to investigate the drug release kinetics [23].

2.5.3. Mucoadhesion Test

The method described by Notario-Pérez et al. [24] was used to evaluate ex vivo mucoadhesion time. A sample of freshly excised veal vaginal mucosa was obtained from a local slaughterhouse and attached to an 8.5 cm × 5 cm stainless steel plate with a cyanoacrylate adhesive. Each tablet was then placed over the mucosa and weight of 500 g was applied for 30 s. The system was placed at

an angle of 60° in a beaker containing 150 mL of SVF and then in the shaking water bath (Selecta® UNITRONIC320 OR, Barcelona, Spain) at 37 °C and 15 opm. All batches were tested in duplicate.

3. Results and Discussion

3.1. Characterization of the Granules

3.1.1. Infrared Spectroscopy

The analysis of the granules by FTIR-ATR, and the subsequent comparison with the spectrum obtained by analyzing the raw materials used in their preparation, reveal any possible interactions that may have occurred at the molecular level during the granulation process. These interactions—if they occur—require an in-depth analysis to ensure that the therapeutic efficacy of the TFV has not been modified.

The two types of Gelucire® analyzed (G39 and G43) showed a very similar FTIR-ATR spectrum, which is to be expected given their close structural similarity (Figure 1B). These spectra are also clearly similar to those obtained by other authors with other types of Gelucire® [25]. Characteristics worth noting include a band at around 1650 cm^{-1} corresponding to the C–O bonds and another double band around 2800 cm^{-1}, due to the C=O bond [26].

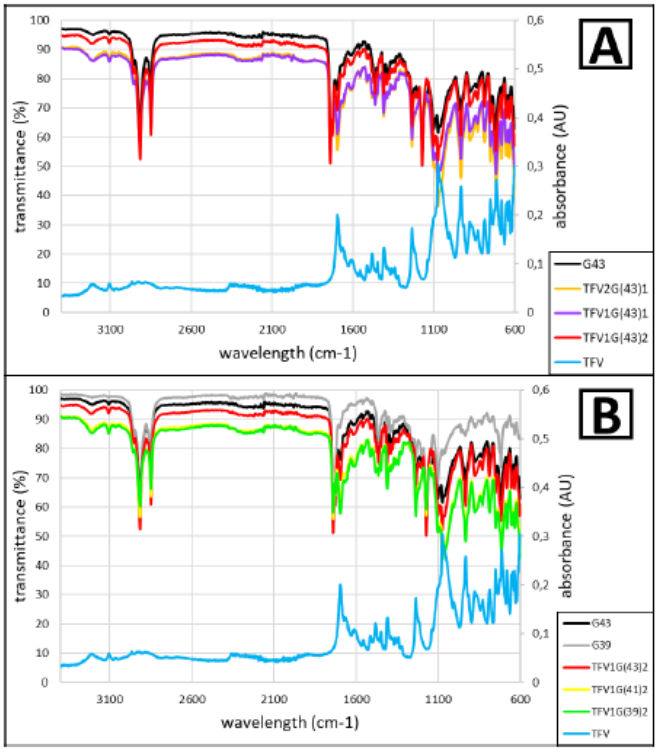

Figure 1. FTIR-ATR spectra of TFV, G43 and their combinations (**A**); and TFV, G43, G39 and their combinations in a 1:2 ratio of TFV/Gelucire® (**B**). The absorbance spectra (right axes) correspond to TFV.

TFV is notable for a band around 1700 cm^{-1}, corresponding to C=O stretching [27]. It also has bands at 3100 cm^{-1} and 3200 cm^{-1} corresponding to C–H and N–H bonds respectively [28] (Figure 1).

The results of the spectra obtained with the prepared granules are in all cases very similar, and always resemble the results that would be obtained by superimposing the spectra for the raw materials (Figure 1).

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The FTIR-ATR analysis confirms that the raw materials—Gelucire® and TFV—do not interact with each other at a molecular level during the granulation process, thus maintaining the drug's properties.

3.1.2. Thermal Analysis

Thermal analysis was performed in order to determine the characteristics of the granules in greater detail. The TGA analysis (Figure 2) shows the weight lost by the granules during heating. The raw materials were also analyzed using this technique. In the case of TFV, about 5% of the weight is lost at around 90 °C (this is probably a loss of the water captured by the drug during storage), followed by a strong degradation that possibly corresponds to the drug decomposition, beginning at around 320 °C. At 500 °C, the sample maintains about 65% of its initial weight. In contrast, the Gelucire® samples show no water loss in the TGA analysis, due to the Gelucire®'s limited uptake of water at ambient humidity conditions [29], and there is no change in the TGA curves until around 240 °C. From this point until 370 °C the samples undergo a marked decomposition and lose over 80% of their weight. With further heating, Gelucire® samples continue losing weight more slowly and when the test ends at 500 °C, less than 5% of the initial weight remains. Both G43 and G39 show similar results, but should be noted that the final weight loss is lower in the G39 sample. These minor differences between the different Gelucire® products can be attributed to the slight variations in their composition [11]. Granules combining TFV and Gelucire® in all cases display an intermediate behavior between the raw materials used in their manufacture. The comparison of granules with different TFV/Gelucire® ratios show that the greater the amount of Gelucire® included in the sample, the higher the final weight loss (Figure 2A). The variations are almost negligible in granules prepared with different Gelucire®, although the inclusion of more G39 than G41 can still be seen to cause the granules to degrade more, as in the case of the raw materials. Finally, it should also be noted that no weight loss is observed in the TGA analysis of the granules at around 90 °C, as occurs in the TFV sample, which confirms that the weight loss is due to water, as the raw materials are heated and the water has already been removed during the preparation of the granules.

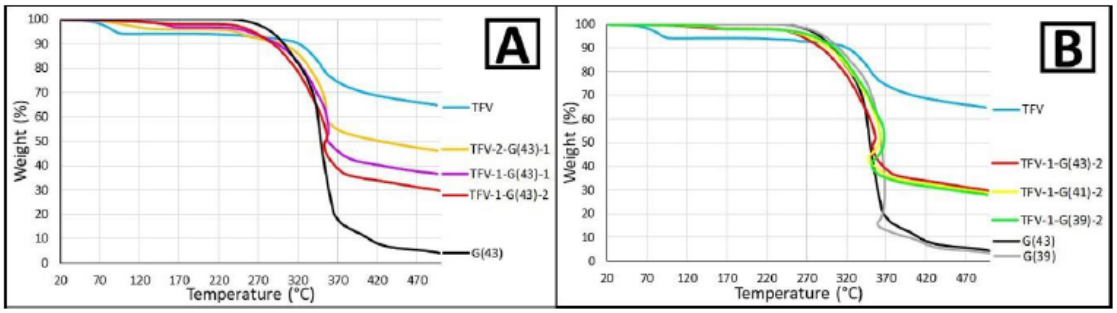

Figure 2. Thermogravimetric analysis (TGA) curves of TFV, G43 and their combinations (**A**); and TFV, G43, G39 and their combinations in a ratio of 1:2 of TFV/Gelucire® (**B**).

DSC analyses were performed to check if during the process of preparation of the solid dispersion it occurs the dissolution of the active principle in the molten vehicle, which would later be reversible by crystallization of the TFV during the cooling of the binary system, and the results are shown in Figure 3. The TFV DSC curve is characteristic, due to an endothermic peak at around 160 °C and an exothermic peak at around 220 °C, which may correspond to changes in the crystallinity of the drug. A stronger endothermic peak beginning at 295 °C corresponds to the TFV melting point [30]. Finally, drug decomposition is observed at around 315 °C.

An endothermic peak can be seen in both G43 and G39 at approximately their respective melting points. These melting points begin shortly after ambient temperature, as has already been observed by other researchers [31], and can be explained by the fact that Gelucire® are multicomponent mixtures with a semi-solid nature. Exothermic readings beginning at 230 °C are observed in both Gelucire®,

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and the main difference is in their decomposition, which in both cases begins after 400 °C but is much more vigorous for G43 than for G39.

When analyzing the DSC curves from the granules combining one or both Gelucire® with TFV, the main observation is an intermediate curve between the curves obtained from the raw materials. The three characteristic peaks of TFV appear in all the prepared granules, so mixing with Gelucire® apparently has no effect on the drug. Nevertheless, it should be noted that the inclusion of TFV appears to lower the melting point of Gelucire® (Figure 3).

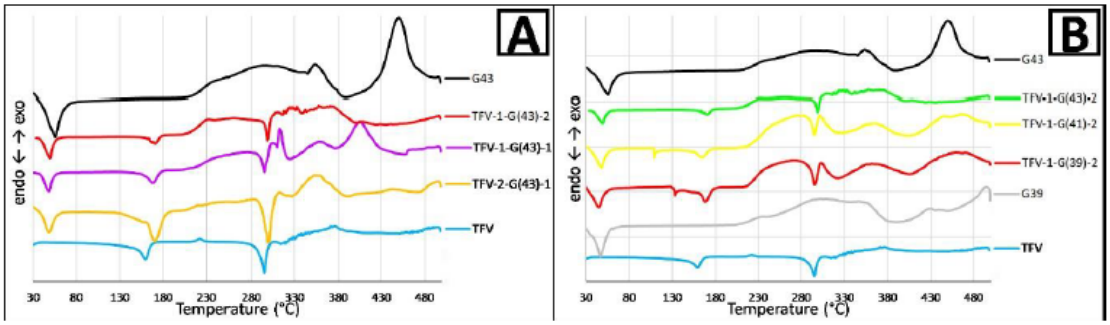

Figure 3. Differential scanning calorimetry (DSC) curves of TFV, G43 and their combinations (**A**); and TFV, G43, G39 and their combinations in a ratio of 1:2 of TFV/Gelucire® (**B**).

Finally, hot stage microscopy (HSM) was performed to complete the thermal analysis of the samples. TFV can be seen as a crystalline substance in the visualization. When heating the sample, some changes in the shape of particles are observed at 167 °C. The temperature continues rising and a similar behavior can be seen on reaching 220°C. These two changes may correspond to crystalline transitions of TFV, as was assumed to occur when analyzing the DSC curve. Finally, the sample begins to melt at around 265 °C.

Both Gelucire® appear as irregularly-shaped particles of a waxy substance with a rough surface. They are crystalline, since polarized light can pass through the samples. The start of the melting process is identified by HSM at a somewhat lower temperature than indicated by the supplier (40 °C for G43 and 36 °C for G39), as shown in the DSC analysis (Figure 3). G39 turns brown at around 310°C, as though the sample had charred, and it appears to start boiling.

Very similar results are obtained when analyzing the granules by HSM. The main changes are summarized in Figure 4. They all appear in the microscope as irregular particles that allow the partial passage of polarized light. In all the samples, the first change observed is the melting of the Gelucire®. The more G39 there is in the granules, the lower the melting point (Figure 4). When comparing samples containing only G41 and TFV, the granules with twice as much TFV as G41 have a higher melting point. However, the melting of G41 is more difficult to visual identify, due to its low proportion, so it probably occurs before it can be visually detected (when a halo of melted Gelucire® is seen around the TFV particles). Once the Gelucire® is completely melted, there are more particles that allow the passage of polarized light, corresponding to TFV crystals surrounded by melted Gelucire®. The first crystalline transition in the TFV raw material is not detected in any of the granules. However, an interesting phenomenon occurs at the temperature of the second crystalline transition of TFV. From 212 °C to 226 °C, depending on the sample, the TFV crystals that allow the passage of polarized light to disappear, probably because after the change in its crystallinity TFV is able to dissolve into the melted Gelucire®. This ability of melted Gelucire® to dissolve drugs has also been noted by other researchers [31]. However, some TFV crystals are still in evidence, so the amount of molten Gelucire® is insufficient to dissolve all the TFV present in the sample. A little later, at between 224 °C and 251°C, the sample carbonizes and turns brown, probably because the dissolved TFV has burned. Between 262–270 °C, the remaining particles that still allow the passage of polarized light disappear completely in some samples, corresponding to the melting of the undissolved TFV, which occurs at the

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temperature at which the TFV raw material melts. Finally, at around 290 °C, the Gelucire® can be seen to be boiling. After analyzing all the information from the thermal analysis of the granules, it can be confirmed that no interaction between Gelucire® and TFV occurs at the manufacturing temperatures; however, a more in-depth study was made using X-ray diffraction techniques to confirm the TFV transitions and some of the interactions that are assumed to occur at higher temperatures.

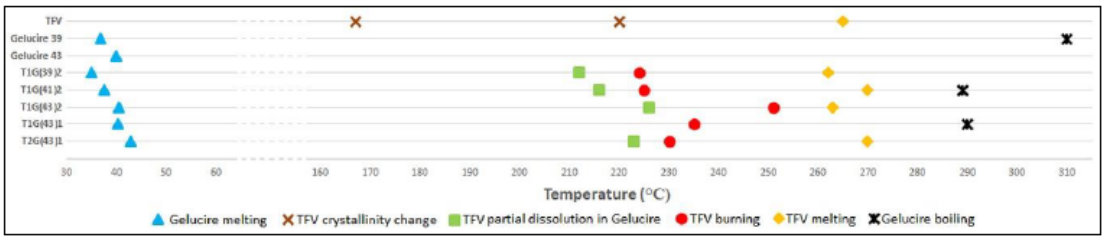

Figure 4. Diagram of the changes visualized during the observation of TFV, Gelucire® and the granules by hot stage microscopy.

3.1.3. X-ray Diffraction

X-ray spectrometry studies were performed to evaluate if these is any change in the crystalline state of the drug during the granulation process. These studies could also corroborate the hypotheses formulated after the thermal analysis of the granules. First the raw materials (TFV, G43 and G39) and the manufactured granules were analyzed to compare them and confirm that there is no interaction between the drug and the carriers during granulation.

The X-ray diffraction pattern for TFV reveals the four characteristic peaks of the drug at 7.3, 14.7, 18.3 and 23.4° 2θ [32]. This clearly confirms that TFV is a crystalline substance, as observed in HSM. There are hardly any differences between G43 and G39, and both are crystalline (as observed in HSM) with two intense peaks at 20.9 and 23.1° 2θ. This agrees with the results obtained for other Gelucire®, which also present peaks at between 20–25° 2θ [33]. A third peak of lower intensity can also be distinguished at around 6.8° 2θ, which is more clearly visible in G39 (Figure 5). The spectra observed in the granules is a mixture of the spectra obtained with their component raw materials, and the lower the proportion of each material in the composition of the granules, the lower the intensity of the characteristic peaks (Figure 5).

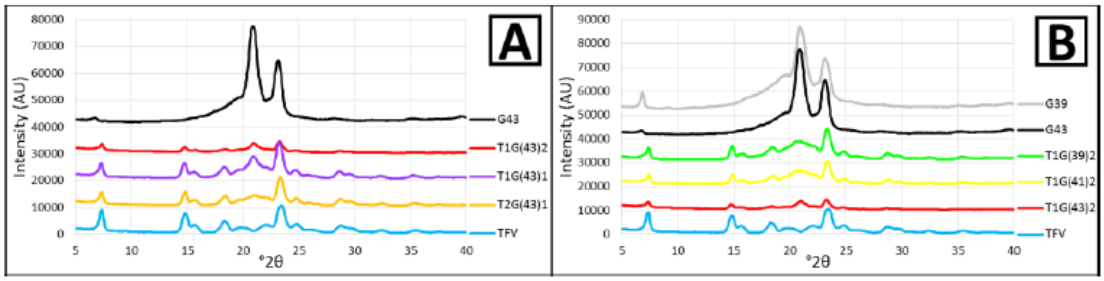

Figure 5. X-ray diffraction patterns of pure TFV, G43 and their combinations (**A**); and TFV, G43, G39 and their combinations in a ratio of 1:2 TFV/Gelucire® (**B**).

In conclusion, this analysis allows us to confirm that no drug-Gelucire® interaction occurs in the granulation process.

However, various changes were observed in TFV in DSC and HSM that have been attributed to the crystalline transition of the drug, although a further study was done to confirm this.

The X-Ray thermodiffraction technique revealed the changes in the drug's crystallinity caused by temperature. A study was done to confirm that the changes observed in DSC and HSM at 167 °C and 220 °C correspond to the crystalline transition of TFV. This consisted of measuring a sample of pure

TFV by X-ray diffraction at room temperature (25 °C) and then after heating. The temperature was maintained for five minutes before and after the temperatures at which changes were observed, then a measurement was taken by X-ray diffraction. After reaching 225 °C, higher than the temperatures at which the previous change had occurred, the sample was cooled and new measurements were taken using the same procedure to determine whether the temperature-related changes are reversible.

The spectra observed when the sample was analyzed at 25 °C is exactly the same as in the previous study at room temperature (Figures 5 and 6). Although other authors have found that the crystallinity of TFV does not change with temperature, their studies reached only 80 °C [32,34]. In our study, the sample was heated to 150 °C before taking the second measurement, prior to the first change in the structure of TFV. Surprisingly, changes can be seen in TFV at this temperature. This is probably because the first transition has already begun, as the characteristic peaks have been displaced to 7.3° and 23.4° 2θ, the peak that was present at 14.7° 2θ has disappeared, and a series of peaks have appeared between 16–19° 2θ. At 175 °C, the spectrum is completely different to the one obtained at 25 °C, with a double peak between 8–9° 2θ, another two double peaks around 17–18° 2θ, and a band with multiple peaks between 19–20° 2θ that were not observed at lower temperatures. This clearly confirms that the first crystalline transition of TFV is complete (Figure 6). At 225 °C the spectrum is slightly different: The peak at 9° 2θ disappears and the intensity of the peak at 17° 2θ decreases. The second crystalline change is therefore complete at 225 °C, allowing the dissolution of TFV in its new state in the melted Gelucire®, as observed in the HSM studies. Finally, the sample was cooled and new measurements were taken, and no change was observed in X-ray diffraction, thus confirming that the crystalline changes in TFV are irreversible.

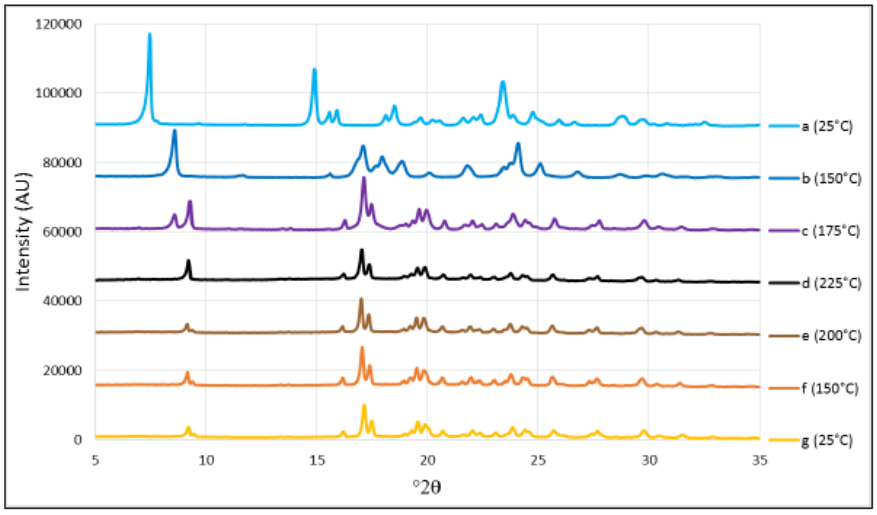

Figure 6. X-ray thermodiffraction patterns of pure TFV while heating to 25 °C (a), 150 °C (b), 175 °C (c) and 225 °C (d); and then while cooling to 200 °C (e), 150 °C (f) and 25 °C (g).

After the IR, thermal and X-ray analysis of the prepared granules, we can confirm that TFV is not altered in the hot-melt granulation process but conserves its therapeutic properties, thus enabling the use of the granules for prevention of HIV.

3.2. Assessment of the Tablets

3.2.1. Swelling Behavior

The evaluation of the swelling behavior of the formulations has a twofold importance: The capture of water is the main mechanism controlling the release of the drug, since it is unable to diffuse until the hydrophilic polymer swells and the water surrounds the granules [35]; and the swelling of the

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formulations can be seen as a way of improving adherence to prophylactic treatment, as the lower water capture would make the tablets more comfortable for women.

The inclusion of the drug granulated with G43 in the polymer matrix clearly improves the formulation in terms of swelling. As can be seen in Figure 7, the maximum swelling ratio is notably lower than in the formulations containing the drug without Gelucire® [16], and especially in the formulations in which HPMC is greater than or equal to CH, due to the predominantly hydrophobic character of Gelucire®. The formulation achieves complete erosion sooner with the addition of the granules: After 216 h instead of the 264 h for the original formulation. This behavior would imply greater adherence to prophylaxis, since the formulation would remain less time in the vaginal environment after the drug release, making it more comfortable for women. The more HPMC there is in the formulation, the higher the maximum swelling ratio. This was expected, as the swelling capacity of HPMC is higher than CH [24]. Finally, no clear differences ca be seen between the formulations containing granules with a different G43/TFV ratio (Figure 7).

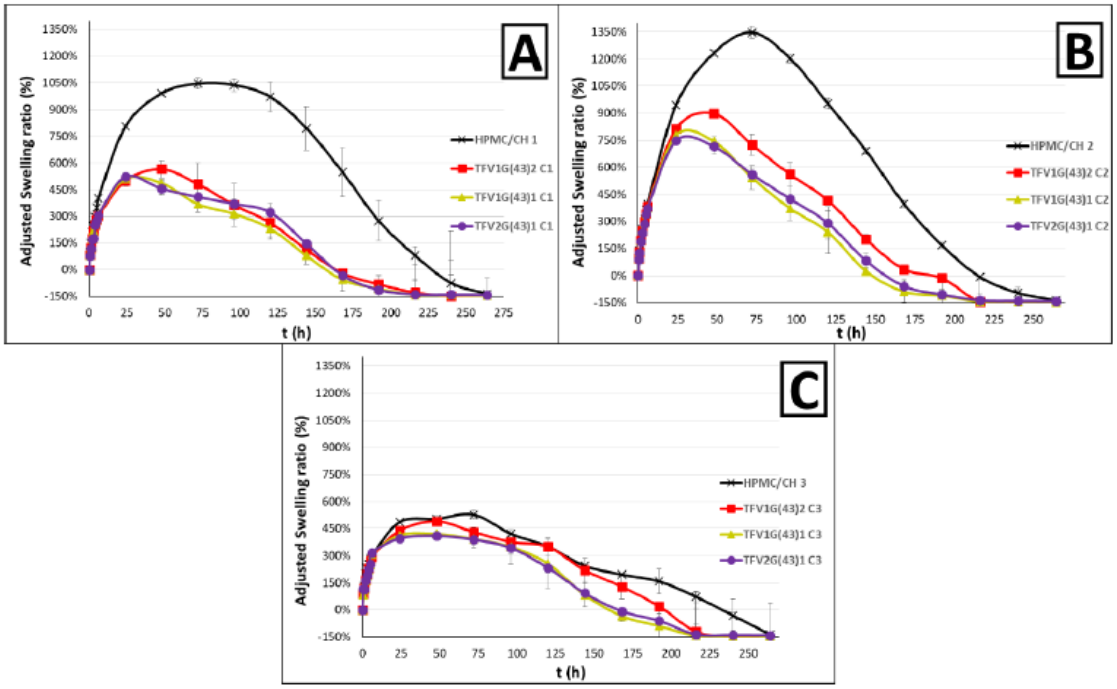

Figure 7. Adjusted swelling ratio profiles obtained from batches containing TFV/G43 drug-loaded granules in HPMC/CH matrices with equal ratios of both polymers (**A**); more HPMC than CH (**B**); and more CH than HPMC (**C**). They are all compared with standard formulations of HPMC/CH [16].

The use of different G39 or a mixture of G39 and G43 does not appear to change the swelling behavior of the formulations. This is to be expected since HPMC and CH are polymers with a capacity to capture water, and both Gelucire® act similarly as a barrier to water diffusion. Only the tablets with more HPMC than CH show a slight difference in the formulations containing G39 (or a G39/G43 mixture), which have a lower water capture than tablets with only G43. Nevertheless, an improvement is again observed compared to the formulations without Gelucire® (Figure 8).

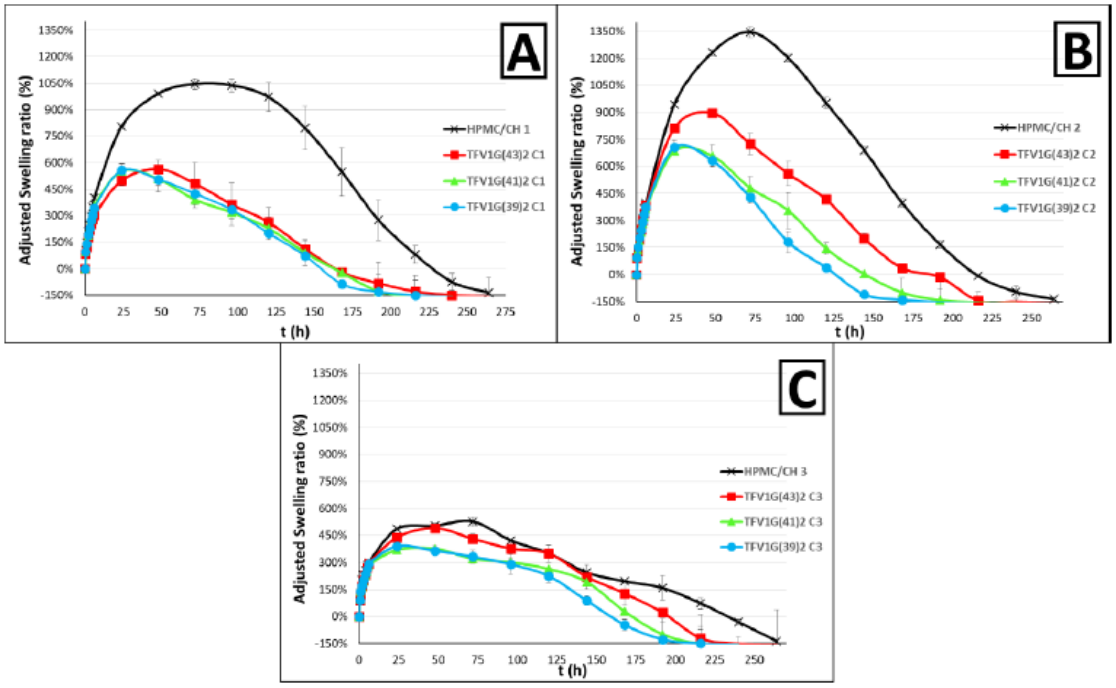

Figure 8. Adjusted swelling ratio profiles obtained from batches containing TFV/Gelucire® (in a 1:2 ratio) granules in HPMC/CH matrices with equal ratios of both polymers (**A**); more HPMC than CH (**B**); and more CH than HPMC (**C**). They are all compared with standard formulations of HPMC/CH [16].

The inclusion of the granules creates barriers within the tablet that hinder the diffusion of water and leads to lower water capture. This not only makes the tablets more comfortable, but would also probably prevent the loss of the drug, since it contains less water through which to diffuse. We can therefore formulate the hypothesis that this formulation would be more effective for the controlled release of TFV, although this must be confirmed in subsequent drug-release studies.

3.2.2. Release Study

As can be seen in Figure 9, the inclusion of mixed TFV/G43 granules in the formulations enhances the controlled release of the drug. These differences can be observed from the beginning of the drug release test, as the Gelucire® must soften as it forms granules with the drug to allow the release of the TFV. This softening is enabled by the presence of water and body temperature. Once the Gelucire® is softened and the drug diffuses through it, it is incorporated in a matrix of HPMC and CH polymers, which form a gel in the vaginal medium through which the drug must diffuse. As has been observed in the swelling test, the presence of the granules hinders the swelling of the polymers, which also causes the drug to be released from the tablet in a more sustained way.

In the samples containing granules with equal or more TFV than G43 (TFV2G(43)1 and TFV1G(43)1), the TFV drug release profiles obtained are quite similar. In these samples the main improvement is the slowdown of the release of TFV, since almost all the drug is released at 120 h (as occurred in the samples that do not use Gelucire®), although there are significant differences in the drug release behavior. In batches with TFV1G(43)2 granules, which include twice the amount of G43 as TFV, the improvement is much more marked (as an example, at 24 h approximately half the amount of drug has been released as in standard batches). This is because of the higher amount of G43 impedes softening and it takes longer for the drug to be released from the granules. Drug release is prolonged to 216–240 h, thus achieving 9−10 days of a controlled release of TFV. This is double the time taken without Gelucire® granules, and could represent a milestone in HIV transmission, since a

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degradable vaginal formulation capable of reaching these controlled release times has never previously been achieved.

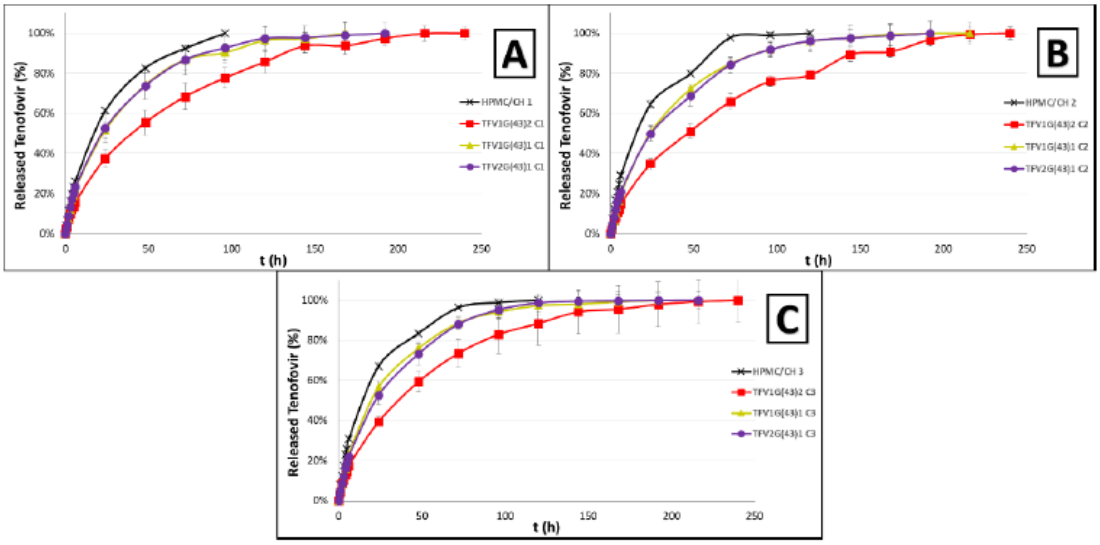

Figure 9. TFV release profiles obtained from batches including TFV/G43 drug-loaded granules in HPMC/CH matrices with equal ratios of both polymers (**A**), more HPMC than CH (**B**) and more CH than HPMC (**C**). All of them are compared with standard formulations of HPMC/CH [16].

It was also necessary to evaluate whether a Gelucire® with a melting point that was closer to body temperature would alter the release of the drug. Figure 10 shows that when G39 or a mixture of G39 and G41 is used to manufacture the granules, TFV is released faster than in tablets with TFV/G43 granules. These batches confirm that the softening of the Gelucire® is a crucial factor for controlling TFV release; this agrees with other authors, who also highlight that in the case of Gelucire® that melt above body temperature, the release of the drug depends on the composition and HLB value [36].

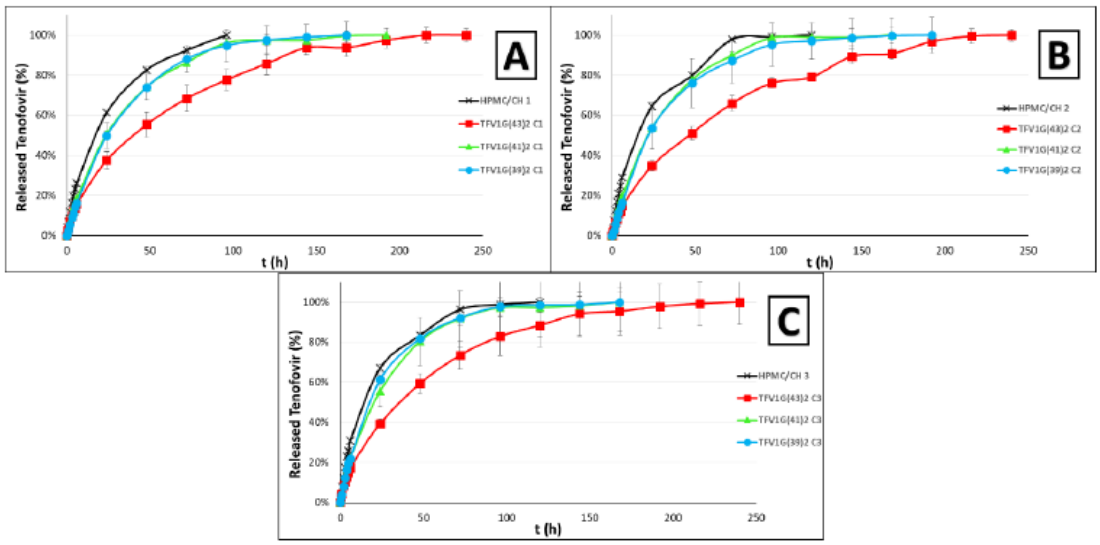

Figure 10. TFV release profiles obtained from batches containing TFV/Gelucire® (in a ratio of 1:2) granules in HPMC/CH matrices with equal ratios of both polymers (**A**); more HPMC than CH (**B**); and more CH than HPMC (**C**). All are compared with standard formulations of HPMC/CH [16].

The TFV1G(43)2 granules are therefore undoubtedly the best formulation. Finally, a comparison must be made of the formulations containing different proportions of HPMC/CH. Although the three proportions have similar TFV release profiles, the formulation with the most HPMC (TFV1G(43)2 C2) shows the greatest controlled release (Figure 10B).

Although the figures visually identify the improvements achieved with the formulations, the f_2 similarity factor was also calculated as a statistical analysis that could demonstrate these differences [37]. Table 3 shows the f_2 values for all the formulations.

Table 3. Similarity factor (f_2) values for the release profiles obtained from reference and problem formulations. Comparisons with significant difference ($f_2 < 65$) are in bold.

REFERENCE	PROBLEM	f_2	REFERENCE	PROBLEM	f_2	REFERENCE	PROBLEM	f_2
C1	T1G(43)2 C1	**44.6**	C1	C2	84.3	T1G(43)2 C1	T1G(43)2 C2	83.4
C1	T1G(43)1 C1	**64.7**	C1	C3	76.9	T1G(43)2 C1	T1G(43)2 C3	78.4
C1	T2G(43)1 C1	66.9	C2	C3	85.6	T1G(43)2 C2	T1G(43)2 C3	68.1
C1	T1G(41)2 C1	**60.4**	T1G(43)2 C1	T1G(43)1 C1	**54.5**	T1G(43)1 C1	T1G(43)1 C2	83.5
C1	T1G(39)2 C1	**60.5**	T1G(43)2 C1	T2G(43)1 C1	**53.7**	T1G(43)1 C1	T1G(43)1 C3	84.9
C2	T1G(43)2 C2	**40.9**	T1G(43)2 C1	T1G(41)2 C1	**56.3**	T1G(43)1 C2	T1G(43)1 C3	75.1
C2	T1G(43)1 C2	**55.8**	T1G(43)2 C1	T1G(39)2 C1	**57.2**	T2G(43)1 C1	T2G(43)1 C2	81.3
C2	T2G(43)1 C2	**56.7**	T1G(43)2 C2	T1G(43)1 C2	**50.0**	T2G(43)1 C1	T2G(43)1 C3	94.5
C2	T1G(41)2 C2	**60.5**	T1G(43)2 C2	T2G(43)1 C2	**51.0**	T2G(43)1 C2	T2G(43)1 C3	84.8
C2	T1G(39)2 C2	**54.7**	T1G(43)2 C2	T1G(41)2 C2	**48.4**	T1G(41)2 C1	T1G(41)2 C2	86.2
C3	T1G(43)2 C3	**43.3**	T1G(43)2 C2	T1G(39)2 C2	**50.0**	T1G(41)2 C1	T1G(41)2 C3	73.7
C3	T1G(43)1 C3	**60.5**	T1G(43)2 C3	T1G(43)1 C3	**54.6**	T1G(41)2 C2	T1G(41)2 C3	83.7
C3	T2G(43)1 C3	**56.5**	T1G(43)2 C3	T2G(43)1 C3	**59.1**	T1G(39)2 C1	T1G(39)2 C2	88.1
C3	T1G(41)2 C3	**61.9**	T1G(43)2 C3	T1G(41)2 C3	**52.4**	T1G(39)2 C1	T1G(39)2 C3	**62.7**
C3	T1G(39)2 C3	**64.1**	T1G(43)2 C3	T1G(39)2 C3	**49.3**	T1G(39)2 C2	T1G(39)2 C3	67.4

The first point to emphasize is that all the formulations developed in the present work improve those with the same proportion of HPMC/CH but without Gelucire® (except for formulation TFV2G(43)1 C1, which has a slight similarity with the formulation without Gelucire®). This highlights the role of Gelucire® in controlling the release of TFV from the tablets. However, when comparing formulations with the same granules but with a different proportion of the HPMC/CH mixture, significant differences were observed in only one case (batches TFV1G(39)2 C1 and TFV1G(39)2 C3). This reaffirms the fact that the combination of HPMC and CH forms a very robust mixed gel capable of delaying the release of TFV regardless of the proportion of polymer [16]. Although the granules' ability to control the release has been demonstrated, these results must be compared with formulations with the same proportion of HPMC/CH but different granules, in order to determine which granules are the most suitable. The comparison of these formulations confirms that the granules identified in Figure 9 as best controlling TFV release (TFV1G(43)2) always present significant differences with the other granules, regardless of the proportion of HPMC/CH in the tablets that include the granules.

The statistical analysis therefore shows that the TFV1G(43)2 granules are the best option for preparing our formulations, although it is impossible to specify the most appropriate proportion of hydrophilic polymers to include in these granules since they are all very similar.

Finally, the experimental data obtained in the drug release tests were adjusted to different mathematical models (zero order, first order, Higuchi, Hixson-Crowell, Hopfenberg and Korsmeyer-Peppas). The correlation coefficients (r^2) obtained after the adjustment are shown in Table 4.

Table 4. Correlation coefficients obtained when experimental data are fitted to different mathematical models.

Batch	Correlation Coefficients (r^2)					
	Zero Order	First Order	Higuchi	Hixson-Crowell	Hopfenberg	Korsmeyer-Peppas
TFV1G(43)2 C1	0.9557	0.7162	0.9975	0.9888	0.9824	0.9915
TFV1G(43)2 C2	0.9658	0.7139	0.9961	0.9925	0.9877	0.9876
TFV1G(43)2 C3	0.9567	0.7188	0.9971	0.9922	0.9858	0.9911
TFV1G(43)1 C1	0.9140	0.6436	0.9885	0.9749	0.9624	0.9795
TFV1G(43)1 C2	0.9298	0.6610	0.9911	0.9884	0.9771	0.9887
TFV1G(43)1 C3	0.9110	0.6514	0.9878	0.9835	0.9690	0.9896
TFV2G(43)1 C1	0.9190	0.6474	0.9920	0.9852	0.9723	0.9801
TFV2G(43)1 C2	0.9351	0.6671	0.9954	0.9911	0.9813	0.9864
TFV2G(43)1 C3	0.9306	0.6849	0.9939	0.9938	0.9834	0.9933
TFV1G(41)2 C1	0.9377	0.663	0.9930	0.9958	0.9877	0.9850
TFV1G(41)2 C2	0.9307	0.6604	0.9912	0.9963	0.9897	0.9889
TFV1G(41)2 C3	0.9148	0.6139	0.9886	0.9918	0.9775	0.9662
TFV1G(39)2 C1	0.9384	0.6522	0.9886	0.9948	0.9850	0.9862
TFV1G(39)2 C2	0.9270	0.6708	0.9854	0.9916	0.9796	0.9941
TFV1G(39)2 C3	0.9005	0.6437	0.9819	0.9879	0.9705	0.9983

As can be seen, all the formulations have a similar drug release profile and a good fit to the kinetics of Higuchi, Hixson-Crowell, Hopfenberg and Korsmeyer-Peppas (Table 4). However, there are slight differences between the batches that merit discussion. One observation is that all batches containing G43 tend to have a better fit to Higuchi kinetics. This model is often used to describe the behavior of matrix tablets, where the drug is released through a diffusion mechanism [38]. The most likely reason that these batches fit this kinetic more closely is that the G43 softens slowly, and the gelation of the outer layer of the hydrophilic polymer has already taken place when it occurs. This explains why the drug release is predominantly controlled by the diffusion mechanism (through both the softened Gelucire® and the gel formed by the HPMC/CH mixture).

The tablets containing the mixture of G43 and G39 have a better fit to the Hixson-Crowell kinetics, a mathematical model applied when the drug is released from the parallel planes of the dosage form, which maintains its shape while its size decreases [39]. In these cases the Gelucire® mixture softens a little faster than in the previous ones; one possible explanation for this drug-release mechanism is that the hydrophilic polymers gel from the outer to the inner layers, and the Gelucire® softens as the vaginal fluid reaches the granules through the gel. This explains why the drug is released in layers, first diffusing the TFV in the outer layers of the tablet, and then the drug in the innermost layers.

Finally, batches with G39 are better fitted to the Korsmeyer-Peppas kinetics (except batch TFV1G(39)2 C1, which has a better fit to the Hixson-Crowell kinetics, although it also fits the Korsmeyer-Peppas kinetics quite well) (Table 4). The Korsmeyer-Peppas model gives us the most information about the drug-release mechanism, since the drug is released in different ways depending on the value of n obtained in the adjustment [40]. Thus for cylindrical tablets, TFV release follows a pure diffusion process ($n \leq 0.45$) or an anomalous transport with simultaneous structural modification and diffusion ($0.45 < n < 0.89$). If there is a structural modification of the polymer matrix, the release is classified as transport case II ($n = 0.89$) or transport Supercase II ($n > 0.89$).

In our case, almost all the formulations can be seen to follow a simultaneous mechanism of structural modification and diffusion (Table 5), which is to be expected since it is first necessary for the hydrophilic polymer gel and the Gelucire® to soften (structural modification) and the TFV to subsequently diffuse before the drug can be released. However, there are three exceptions which present a Supercase II release and are precisely the three that include G39 in the granules (Table 5). In these cases, the melting temperature of the vehicle is so low that the G39 softens as soon as it is at body temperature, even before the vaginal fluid reaches the granules, implying that the structure of the tablet changes even before the HPMC/CH mixture begins to gel.

Table 5. TFV release kinetics from the different batches showing the kinetic constants for the models with the best fit.

Batch	Higuchi	Hixson-Crowell	Hopfenberg	Korsmeyer-Peppas	
	K_H	K_{HC}	K_{HF}	K_{KP}	n
TFV1G(43)2 C1	0.083	0.0040	0.0054	0.042	0.68
TFV1G(43)2 C2	0.080	0.0039	0.0052	0.036	0.71
TFV1G(43)2 C3	0.088	0.0045	0.0060	0.046	0.67
TFV1G(43)1 C1	0.102	0.0059	0.0075	0.045	0.82
TFV1G(43)1 C2	0.103	0.0060	0.0076	0.037	0.86
TFV1G(43)1 C3	0.106	0.0065	0.0081	0.048	0.81
TFV2G(43)1 C1	0.103	0.0061	0.0077	0.056	0.73
TFV2G(43)1 C2	0.108	0.0058	0.0074	0.047	0.78
TFV2G(43)1 C3	0.105	0.0065	0.0081	0.052	0.75
TFV1G(41)2 C1	0.107	0.0067	0.0083	0.038	0.85
TFV1G(41)2 C2	0.111	0.0076	0.0091	0.040	0.86
TFV1G(41)2 C3	0.110	0.0073	0.0089	0.043	0.88
TFV1G(39)2 C1	0.108	0.0066	0.0083	0.026	0.97
TFV1G(39)2 C2	0.109	0.0067	0.0084	0.029	0.93
TFV1G(39)2 C3	0.112	0.0075	0.0091	0.042	0.93

In addition to providing an insight into the mechanism of TFV release, this adjustment allows us to identify which formulation best controls the drug release based on the values of the release constants (K_H, K_{HC}, K_{HF} and K_{KP}). A comparison of these constants conclusively highlights the batches with the TFV1(G43)2 granules previously identified as the most suitable for controlled-release formulations. Taking the Higuchi kinetic as an example, which has the best fit for these formulations (Table 4), the batches that include these granules have a K_H value of 0.08, while the K_H value of the other batches is always greater than 0.1 (Table 5). This points to the greater control over the release of the TFV from these batches, but once again makes it impossible to select the ideal proportion of HPMC/CH, given the close similarity of the constants obtained with batches C1, C2 and C3.

3.2.3. Mucoadhesion Test

Finally, another crucial parameter to evaluate was the mucoadhesion residence time of the formulations. Mucoadhesion is important because the tablets must remain adhered to the vaginal mucosa during the time TFV is being released [41]. The detachment of the formulation would imply an incomplete prophylactic treatment and discomfort for women, leading to a decrease in the use of the formulations.

A comparison of formulations with different ratios of TFV/G43 reveals that the more G43 the formulation contains, the longer it remains adhered to the vaginal mucosa (Figure 11). As this excipient has no adhesive properties this bioadhesion cannot be directly attributed to G43, so the only possible explanation is that it acts as a structural agent that helps form a more robust gel—as observed in the swelling test (Figure 7)—and hence remains attached for longer. In terms of the influence of the type of Gelucire®, it can be seen that the lower the melting point of the Gelucire®, the shorter the mucoadhesion time. This is probably due to the easier and faster softening of G39, which again causes the tablets to erode faster. Finally, the C2 batches appear to be the most mucoadhesive, undoubtedly due to the higher mucoadhesion of HPMC than CH [24].

TFV1G(43)2 granules therefore confer the strongest adhesive properties on the tablets. Among these batches, it is worth highlighting TFV1G(43)2 C2, which remains adhered to the vaginal mucosa for almost 225 h.

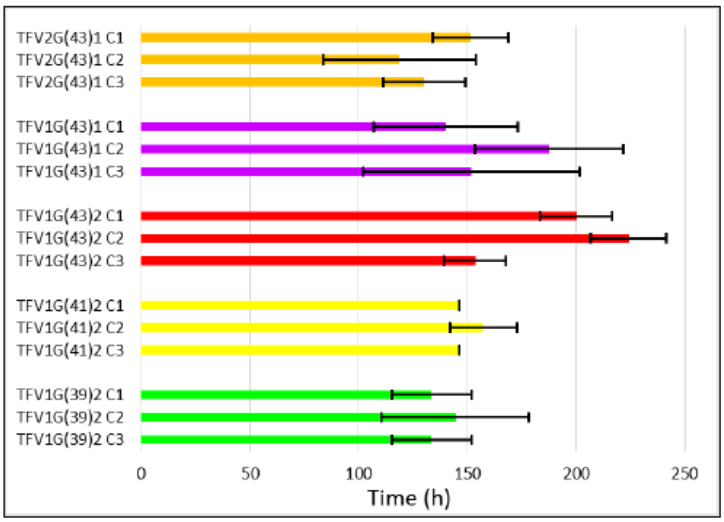

Figure 11. Mucoadhesion residence time in the simulated vaginal fluid in the batches.

The results of these studies, which are summarized in Figure 12, can be used to select the optimal formulation for preventing the sexual transmission of HIV.

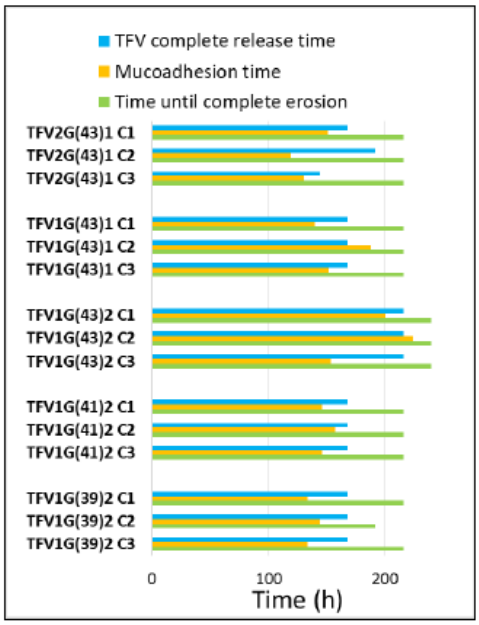

Figure 12. Summary of the data obtained from the drug release, mucoadhesion and swelling test. TFV complete release time (blue), mucoadhesion time (orange) and time until complete erosion (green) are shown for each batch.

The main parameter is undoubtedly the time taken to release the TFV from the formulation, since the aim of this work was to develop weekly sustained-release tablets whose administration could be markedly spaced in order to improve women's adherence to prophylactic treatment, which is currently the main problem with vaginal microbicides. Batches containing TFV1G(43)2 granules achieve the longest controlled release of TFV (216h), above the established target. However, the objective was not only to achieve a longer release time, but also to ensure the mucoadhesion of the formulation for as long as the drug is being released; there are only two formulations whose mucoadhesion time exceeds

their complete TFV release time (batches TFV1G(43)1 C2 and TFV1G(43)2 C2). Finally, the formulation must not remain for long in the vaginal environment after the complete TFV release.

Batch TFV1G(43)2 C2 is conclusively selected as the optimal formulation for HIV prevention, since it is able to release TFV in a sustained manner for 216 h and remains attached to the vaginal mucosa throughout. Almost all the formulation has been eroded by the end of the process (with complete disintegration at 240 h), making it comfortable for women and enabling the next dose of the prophylactic treatment to be easily administered.

4. Conclusions

The combination of hydrophobic granules (prepared with Gelucire®) and hydrophilic matrices (HPMC and CH) in the development of vaginal mucoadhesive tablets allow the sustained-release of TFV.

The ratio TFV-Gelucire, as well as the proportion of HPMC and CH is critical for the development of the optimal formulation. Thus, the one with the best results contains TFV1G(43)2 granules in a mixture of HPMC and CH in a ratio of 1.9:1, which allows the sustained release of TFV for 216 h. The formulation remains adhered to the vaginal mucosa throughout this time, so a weekly administration of the tablets could protect women again the sexual transmission of HIV.

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CAPÍTULO VI

DESIGN, FABRICATION AND CHARACTERISATION OF DRUG-LOADED VAGINAL FILMS: STATE-OF-THE-ART

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Review article

Design, fabrication and characterisation of drug-loaded vaginal films: State-of-the-art

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ABSTRACT

Films have undoubtedly seen the most significant advances in their development in recent years of all the pharmaceutical forms for the vaginal administration of drugs. Films combine the advantages of gels and solid pharmaceutical forms, and their great versatility is largely determined by the numerous polymers that can be used for their fabrication. They may be based on many natural polymers, and cellulose derivatives, polyvinyl alcohol or acrylic derivatives – among others – are also frequently used. The combination of different polymers and the inclusion of plasticizing agents makes them extremely versatility for responding to a range of therapeutic needs. The techniques used to produce films have also undergone substantial development. Although the solvent casting technique is by far the most widely used in fabrication, alternative options have also emerged such as electrospinning, moulding extrusion and 3D printing. Various research groups have proposed a proliferation of assays to characterise vaginal films in recent years, which highlight the importance of the preliminary evaluation and determination of the films' uniformity, in addition to tests to determine their permeability and hydrophilic/hydrophobic coefficient and their mechanical properties, the application of imaging techniques and thermal analysis, and especially the evaluation of the mucoadhesive properties and control over the drug release. This article offers a critical overview of the advances in the development and fabrication of films intended for vaginal drug delivery, and summarises current clinical applications for vaginal films.

1. Introduction

The study of different pharmaceutical forms allows the therapy to be adapted to the characteristics required by either the active compounds or the patients. Although they are still far from commonly used, one pharmaceutical form that has gained in importance in recent years is vaginal films. According to the more traditional definition, a film is a thin sheet, typically formed by one or several polymers, in which the active substance is dissolved or dispersed, and characterised by a rapid dissolution or disintegration in the presence of body fluids [1–3]. The composition of the films may include matrix-forming materials (mostly polymers), plasticisers (usually indispensable for providing adequate mechanical and organoleptic properties), active ingredients, residual solvents (typically water), and other substances that may act as stabilizers, disintegrants or preservatives [4,5]. The main advantages of vaginal films compared to other dosage forms are their small size and thickness, and the fact that they are easy and economical to manufacture and can be administered conveniently without the need for an applicator [6–9]. Compared to gels, they avoid leakage and messiness, and microbial contamination is reduced as the water content is minimal [10–12], meaning they can be used to stabilize drugs that degrade in aqueous conditions [13,14]. Films are also notable for their adhesiveness due to their low weight, large surface area and the inherent properties of the matrix-forming polymers in their composition [15–18]. They have also been used as vehicles for drug-loaded nanocarriers. One advantage is that they may prevent drug release from nanoparticles during storage, which usually occurs in aqueous-based systems such as gels [19].

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The origin of films as drug dosage forms can be traced to their use for buccal administration, when they were developed for food applications (*e.g.* breath mints). Their use rapidly spread to other research areas including cosmetics and nutraceutical products, and they ceased to be used exclusively for buccal administration to become the first films for topical (skin) application [11]. More recently, the pharmaceutical industry has developed sublingual, gastro-retentive and implantable films, as well as systems intended for other types of mucosal administration beyond the buccal mucosa, such as the vaginal mucosa [1]. Films are also currently being used for new applications such as food packaging, where they have been adopted by the food industry as a food preservation solution for coatings and for the development of films loaded with antimicrobial drugs to improve food preservation [20]. All the knowledge acquired in this field is perfectly applicable to the preparation and characterisation of drug-loaded films, since the physicochemical and technological factors analysed are essentially the same [21]. The first vaginal films were developed for contraceptive purposes [22], but formulations are being developed for many other applications [23,24]. Vaginal films have traditionally been formulated for rapid dissolution after administration, but more research is also being done to develop vaginal films that slowly disintegrate, or to control drug diffusion in order to achieve sustained drug release [3,5,12].

There are many considerations to bear in mind when designing a pharmaceutical dosage form for vaginal administration [11]. First, it must be considered that the vagina poses several challenges for the administration of drugs. This natural mucosal cavity is a thin fibromuscular tube, whose length typically ranges from 7 to 10 cm, formed by four different layers of cells; stratified squamous epithelium, lamina propria, muscular and adventitia layers. The vaginal epithelium, whose thickness can vary from 0.2 to 0.5 mm, constitutes the main barrier to drug absorption, and in many instances determines drug efficacy and toxicity [25]. Mucus produced by cervical epithelial cells and the vaginal fluid (a complex mixture of mucus and other components that is present in the vagina) protect against the entry of pathogens. Cervicovaginal fluids serve as a dissolution medium for active ingredients but can also pose an important barrier to the transport of drugs and drug carriers [26]. These fluids also contain components that can interact with drugs and modify their activity. The vaginal pH of women in their reproductive years is usually between 3.5 and 5, but may be affected by certain diseases. Ideally, ingredients from a film formulation should not affect this pH.

Hydrophilic films, once administered, require hydration in the vaginal environment to form a gel and dissolve or disintegrate. The amount of fluid absorbed by the film, and the speed at which it is captured, will determine the rheological characteristics of the gel [6]. The small amount of fluid available in the vagina can hinder this process. Indeed, it may be impossible to achieve homogeneous drug distribution, which may decrease the effectiveness of the formulation. Vaginal discharge is increased during menstruation or in certain local infections, and this can also modify the retention of the formulations and cause variations in the effectiveness of the treatment [7]. Mucoadhesive formulations, which can remain in the vaginal environment even under these conditions, may offer a way to overcome these problems. Finally, the vaginal microbiota, which should ideally remain unchanged in the presence of the film, is another factor that must be considered in the design of vaginal films [1,11].

There are currently three vaginal films on the market: a contraceptive film, a vaginal lubricant and a scented vaginal film [13]. According to some studies, films are generally well accepted by women and even preferred by some compared to other pharmaceutical dosage forms, due to their small size, comfort, ease of application, storage, handling and portability [11,14]. This is particularly relevant considering that the vaginal administration of drugs is often limited by poor adherence by users. The characteristics of the dosage forms greatly influence this aspect. For example, gels or creams can cause leakage and messiness, thus leading users to discontinue the treatment. Some women prefer films over gels because of their aesthetic appeal [27]. In terms of the films' characteristics, women prefer square, 2 × 2 inch, translucent, smooth, thin products [2]. One feature that women demand from films is that they must be odourless and tasteless, largely due to their possible use during sexual intercourse. In terms of application, a slightly greater proportion of women prefer an applicator to those who prefer to administer the film only with their fingers [28]. One common problem with films is that they start dissolving/disintegrating immediately after introduction in the vagina, causing them to stick to the users' fingers.

This article provides a detailed review of the most important factors in the classification, fabrication – including the description of the most commonly used techniques and ingredients – and the typical characterisation assays of vaginal films. It also gives a brief overview of their clinical use.

2. Classification

The numerous possibilities in regard to the techniques and raw materials that can be used to obtain films allow them to be classified according to different parameters, namely solubility, structure and drug-release mechanism. These are described below.

2.1. Based on the film solubility

Hydrophilic films are characterised by undergoing rapid dissolution in the presence of aqueous media such as vaginal fluid [29]. They are especially useful when rapid release of the active substance is intended. These films are prepared with hydrophilic polymers, and the film structure is designed to be as thin and porous as possible in order to accelerate water influx and the dissolution or dispersion of the film matrix. The incorporation of polar plasticisers usually helps dissolve the polymer film; the plasticiser also dissolves in the presence of the aqueous medium and leaves gaps in the film structure, meaning that the polymer is more exposed to the medium, which accelerates its dissolution. The vast majority of the vaginal films in the literature and the films available on the market fall into this category. Also worth noting are films formulated with polymers which do not immediately dissolve in the presence of liquid medium but undergo gelation [7]; these films can be regarded as an alternative to vaginal gels.

Non-water-soluble films have also attracted increasing interest in recent years. They have been proposed as a means of prolonging the residence time of the formulation and the drug release time. Drug release occurs *via* the diffusion mechanism [20]. They are fabricated using hydrophobic or amphiphilic polymers (with a predominance of the hydrophobic part). Examples include films made with zein, ethyl cellulose or some acrylic acid derivatives [24,30–33].

2.2. Based on the film structure

Films with only one polymer layer are the simplest and most common design, and may contain one or more matrix-forming materials. Films obtained from a single film-forming ingredient are becoming less frequent as it is more difficult to ensure the adequate final properties for technological and therapeutic needs (Fig. 1a). Their properties are controlled by the film thickness and the addition of plasticizing substances [9,20,34,35].

The most common strategy today is to obtain blend films combining two or more matrix-forming materials (Fig. 1b). Blend films are frequently developed to improve films' mechanical properties with a single polymer. The formation of bonds between the chains in the different polymers creates a more flexible and robust three-dimensional structure [21,36]. By adjusting the proportion of each polymer in the mixture, the film's properties can be modified according to the characteristics of mechanical strength, thermal stability, permeability or moisture

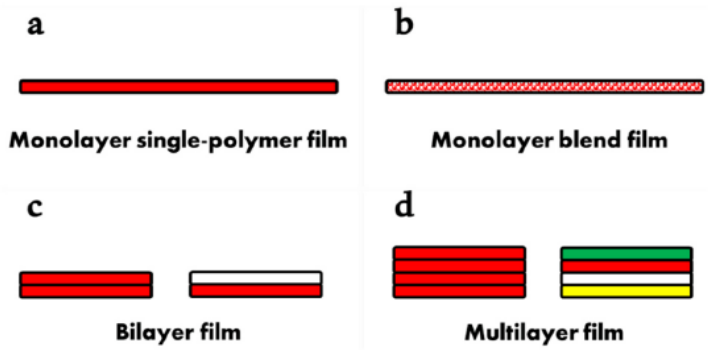

Fig. 1. Structure of monolayer single-polymer films (a), monolayer blend films (b), bilayer films (c) and multilayer films (d). Each colour represents a different ingredient. Blend films combine several ingredients in the same layer (red and white colours). Examples of bilayer and multilayer films show systems based on layers obtained from the same polymer (red colour) or different ones (multicolour). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

resistance desired [37,38]. The combination of hydrophilic and hydrophobic (or amphiphilic) polymers is becoming increasingly common [24] as a means of combining the advantages of hydrophilic and hydrophobic films [1]. When mixed films are obtained, it is important to verify that the polymers do not undergo a separate phase transition, since this would indicate a poor interaction between the formulation's components [39].

Multilayer films offer an attractive drug delivery platform, as they allow the properties of each layer to be combined separately, thus giving these systems greater versatility (Fig. 1c and d) [40]. *In situ* bilayer films can be obtained by preparing one solution with the two polymers (they must have very different precipitation rates to ensure that one forms a polymer layer while the other remains in solution) [41]. However, the most common way to achieve bi- and multilayer films is through the layer-by-layer technique, which involves the sequential preparation of two or more polymeric layers with any of the methods previously described for the preparation of monolayer films; the solvent casting method is the most commonly used [42]. Good compatibility between the polymers in both layers is essential to ensure they do not separate.

In the field of drug release system development, several layers with different properties can be combined to modulate and optimize drug-release processes. It is increasingly common to prepare bilayer films in which the outer layers that are in direct contact with the mucosa are mucoadhesive, while the inner layers control the drug release [42]. Cazorla-Luna et al. [43] developed polyelectrolyte bilayer films with the layer-by-layer technique for vaginal use as a microbicide. One layer consists of chitosan derivatives, which is highly mucoadhesive and has bactericidal properties, and the other layer is formed of Eudragit® S100, which is capable of controlling the release of tenofovir (Fig. 2).

Another method used to obtain bilayer vaginal films is to prepare two individual drug-loaded films of the same size, which are then compressed together using a press [18]. This design has proved effective in modulating the release of two microbicide drugs, namely tenofovir disoproxil fumarate and emtricitabine, to prevent the transmission of human immunodeficiency virus (HIV). Finally, it is also worth mentioning the multilayer hydroxypropyl methyl cellulose (HPMC) films prepared by Zhang et al. by stacking different films using a HPMC gel as a binding material between the different layers [44]. This technique could also be applied in the fabrication of vaginal films (Fig. 3).

2.3. Based on the drug release mechanism: smart films

In the last few decades, so-called "stimuli-responsive" or "smart" polymers have been widely studied. The physicochemical properties and/or structural conformation of these materials can be modified in response to stimuli from the surrounding environment [45], so they can be used for the controlled release of drugs [46]. The stimuli able to affect the characteristics of smart polymers include physical stimuli such as temperature, light, ultrasound or magnetic field, chemical stimuli such as pH, ionic strength or solvent, and biological stimuli such as enzymes, glucose and antigens [45]. pH-responsive films can be obtained by the layer-by-layer technique described previously, which even allows the preparation of films that are sensitive to multiple stimuli [47].

pH-sensitive polymers are the most intensively explored stimuli-responsive polymers in drug delivery, since different pH values can be found in physiological conditions [46,48]. pH-responsive polymers contain ionizable groups with an acidic or basic character that are attached to the backbone chain and cause the molecule to donate or accept hydrogen ions from the medium in response to variations in pH. This ion exchange can disturb the hydrodynamic volume, chain

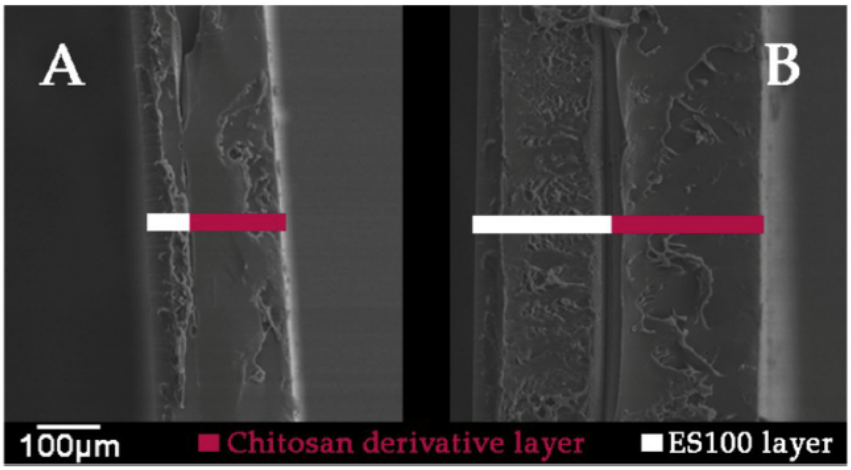

Fig. 2. Micrographs of the cross-section of layer-by-layer films at 100 times magnification, with 75 mg of Eudragit® S 100 (ES100) (A) or 150 mg of Eudragit® S 100 (B) and a chitosan derivative. Reprinted from [43] under the terms of the Creative Commons Attribution License 4.0 (Copyright 2020 Cazorla-Luna et al.; doi: https://doi.org/10.3390/md18010044).

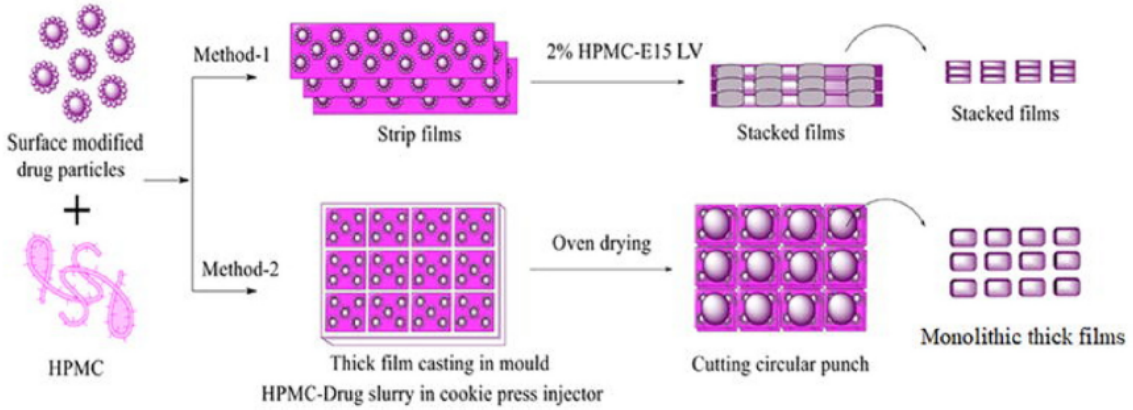

Fig. 3. Schematic of fabrication methods for stacked films (Method-1) and monolithic thick films (Method-2). Reprinted from [44], Copyright (2018), with permission from Elsevier.

conformation and solubility of the polymer, producing the release of the drug from the polymeric system [49]. The release may occur due to the dissociation of the drug from the carrier or to the structural modification of the polymer, since the drug can be loaded either by linking or by physical entrapment [50].

The pH-responsive polymers used in the formulation of vaginal systems with pH-dependant drug release include anionic polymers that are mainly based on acrylic acid, such as Eudragit® [46,49]. Cationic polymers, mostly containing chitosan, have also been studied in the fabrication of pH-responsive vaginal dosage forms [46,51]. Changes in acidic vaginal pH are frequent, often rising to neutral or alkaline values in situations such as menstruation, pregnancy, pre- and post-menarche, or semen deposition after sexual intercourse. pH-sensitive films comprising responsive polymers are an interesting approach to modulate drug release in the vagina (Fig. 4). Prominent among them are semen-triggered vaginal formulations that are specifically intended for the prevention of sexually transmitted diseases [49]. Another application of pH-sensitive vaginal dosage forms is to protect drugs that can be degraded by acidic vaginal pH (such as proteins) [49,52].

A 2006 patent covers pH-responsive vaginal films fabricated by solvent casting. The release of the drug occurred at a pH of equal to or over 7, when the pH-sensitive polymer becomes electronically neutral. This behaviour is interesting since these pH values are reached in the presence of semen and are related to many vaginal diseases [51].

Janovák et al. developed pH-sensitive films for the treatment of vaginal diseases. These films were prepared by the spray-coating method and consisted of one layer of silver nanoparticles and one polymer layer based on pH-sensitive copolymers of methacrylic acid and butyl-methacrylate obtained by photopolymerisation. The polymer layer of the resulting films swelled at pH 4.5–5 due to the electrostatic repulsion between the ionized carboxylic groups and the weakening of the hydrophobic interactions between the butyl-methacrylate units at this pH. This layer dissolved at a pH over 5, causing the release of the nanoparticles, which would be effective only at the pH values often associated with vaginal infections [48]. Our research group has also formulated polyelectrolyte layer-by-layer films consisting of a mucoadhesive layer formed by a chitosan derivative (chitosan lactate, chitosan tartrate or chitosan citrate) and a pH-responsive layer based on Eudragit® S100. These films showed controlled release of tenofovir in simulated vaginal fluid for up to 5 days and a fast release of the drug in the presence of simulated seminal fluid from 4 to 6 h [43].

Modified polyurethanes have also been proposed as pH-responsive polymers for producing vaginal films. Kim et al. synthesized polyether-polyurethane copolymers to fabricate vaginal membranes by solvent casting. The formulation showed pH-responsive and reversible surface modifications that only allow drug release at pH 7 [53]. This research group also prepared nanofibrous membranes through electrospinning. Again, the system was able to hold the release of drugs at vaginal pH and release the loaded nanoparticles at pH 7 [54]. Solanki and Thakore also synthesized pH-sensitive polyurethanes to fabricate films by the solvent evaporation method. The higher the pH of the medium, the greater the swelling of the films, which degraded under highly alkaline conditions [46]. In view of their pH-dependant characteristics, these films would be a promising option for the development of pH-sensitive vaginal films as microbicides.

3. Film ingredients and fabrication

The variety of ingredients that are included in vaginal films is growing rapidly, especially due to the fabrication of polymer-based films that had never previously been considered for this administration route. Although the pharmaceutical industry's experience of manufacturing drug-loaded films is not as extensive as for other dosage forms, the methods to obtain these films are already well established.

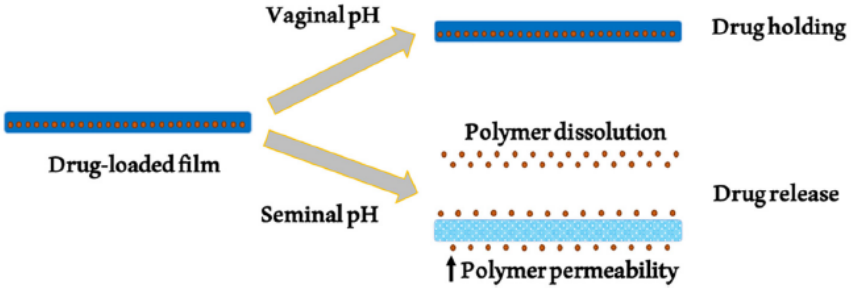

Fig. 4. Drug release behaviour in pH-responsive vaginal films. Dark blue represents a film which is not affected by the medium. Light blue represents a film that has been modified regarding its permeability to the medium. Red dots represent drug molecules; when red dots are incorporated into the film, it denotes that the system does not release the active principle. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.1. Film-forming ingredients

The matrix-forming ingredients must have good mechanical properties and sufficient peel strength [1]. The choice of substances used to fabricate the films will affect the drug release, and there is a choice of rapidly wetting and spreadable film-forming ingredients – which release the active substance quickly –, and others with better barrier properties when the aim is to isolate the drug to prolong its release. Several polymers have already been evaluated for the fabrication of vaginal films, and there is no doubt that films made with other substances will appear in the future. According to their origin and production method, they can be classified as natural, semisynthetic or synthetic.

Natural matrix-forming ingredients have been widely studied and can be obtained from several sources, mainly plants but also animals, fungi or bacteria. Almost all are hydrophilic, although the time taken to dissolve or form a gel in the presence of an aqueous medium can differ significantly. Hydrophilic polymers may also be useful to provide suitable adhesion to mucosa [55]. Nevertheless, zein is a natural and non water-soluble substance with good film-forming applications. Natural film-forming ingredients that have been used to fabricate vaginal films are summarised in Table 1. Nevertheless, many other natural macromolecules have been applied in films for non-vaginal use (guar gum, locust bean gum, konjac glucomannan, gum arabic, karaya gum, starch, hyaluronic acid, agarose, ulvan and curdlan) and they should not be ruled out as candidates for the fabrication of vaginal films in the near future [56–65].

Cellulose derivatives are semisynthetic excipients that are widely used by the pharmaceutical industry and are especially useful for their excellent film-forming properties [75]. Without question, the most frequent cellulose derivative used for manufacturing vaginal films is HPMC, a non-ionic polymer characterised for being water soluble and biodegradable [67]. The molecular weight of the cellulose derivative is very important; the lower the molecular weight, the lower its viscosity and film-forming ability. A higher molecular weight gives the structure more resistance and a better ability to control drug release [76]. Films are generally obtained from the dissolution or suspension of HPMC at room temperature or in warm water [68,72]. Nevertheless, there are also examples of HPMC films prepared with organic solvents, such as the mixture of methanol and dichloromethane [9]. Other cellulose derivatives (characterised for being water-soluble and having very similar properties to HPMC) that have also been used to fabricate films are hydroxypropyl cellulose (HPC), hydroxyethyl cellulose (HEC) and carboxymethyl cellulose [27,71,77,78]. Although these films tend to have quite good mechanical properties, polar plasticisers like propylene glycol, polyethylene glycol or glycerol can be added [13,24,74].

Ethyl cellulose is characterised for being insoluble in water and soluble in organic solvents (such as ethyl acetate) [74,79], so its main advantage is the waterproofing it provides, although it also has excellent mechanical properties [21]. It is commonly used in the pharmaceutical industry to obtain coatings and for encapsulation [39]. It can be dissolved in organic solvents to prepare hydrophobic films. It is usually applied to achieve sustained release in coatings of solid forms, and would be very useful for fabricating films for the prolonged release of drugs [41]. As with the HPMC, the mechanical properties of the films will vary according to the viscosity and molecular weight of the ethyl cellulose [16].

Among the synthetic polymers used for fabricating vaginal films it is worth highlighting polyvinyl alcohol (PVA). This polymer is derived from polyvinyl acetate and is the most commonly used for the preparation of rapidly dissolving vaginal films [80]. It is notable for its high biocompatibility and is considered highly suitable for vaginal administration due to its pH and osmolarity in solution, which are similar to those commonly found in the vaginal environment. The PVA is dissolved in hot water (90–95 °C) [13,74] to form a translucent, odourless, homogeneous soft film [18]. However, due to its low elasticity it is commonly associated with other polymers to obtain mixed films, frequently cellulose derivatives [19,81]. The plasticisers with the best compatibility with PVA are polyols, such as glycerol or polyethylene glycol [38,82].

Polyvinyl pyrrolidone (PVP) is another synthetic polymer that is biocompatible and has good film-forming properties, and stands out for being soluble in organic solvents and water, so PVP films can be prepared from both solutions. It also facilitates the combination of PVP with almost any polymer, so it is very easy to find references to blend films including this polymer [16]. Mixed films of PVA and PVP are especially common, since they are perfectly compatible and complement each other's properties [83].

Finally, acrylic derivatives are other film-forming substances that have been used to prepare vaginal films. Carbomer is a hydrophilic polymer whose structure is formed mainly by acrylic acid, which is notable for its high capacity to capture water and its bioadhesive properties [68], which allows the polymer solution used in the films to be prepared in water. Polar plasticisers such as polyethylene glycol are frequently incorporated to improve flexibility and avoid brittleness in the films [8]. Although the solution can evaporate in ambient conditions, the temperature can be increased up to 60 °C to accelerate the process [10]. Polymethacrylates (mainly the polymers marketed as Eudragit®) are often used for controlled drug-release films. These are hydrophobic polymers that are soluble in organic solvents or alcoholic solutions, many of which have a pH-dependent behaviour. They are rarely used alone, but as part of blend films to control drug release or improve the resistance and decrease the permeability of other polymers. They are also often used in multilayer films, in which the Eudragit® layer is incorporated to control the release of the drug [43]. A few of them require the incorporation of plasticisers to ensure good mechanical properties. As these polymers are poorly water-soluble, they have excellent compatibility with amphiphilic plasticisers such as citrates (*e.g.* tributyl citrate, triethyl citrate) or fatty acids (*e.g.* oleic acid).

3.2. Plasticisers

The addition of plasticizing substances to the films is essential when their mechanical properties are poor. Typically, plasticisers for film fabrication are small organic molecules with high boiling temperatures [84]. The ideal plasticiser can be inserted between the polymer network, thus decreasing the tension between the chains [34]. It acts by reducing intra- and intermolecular interactions between polymer molecules, causing a decrease in the glass transition temperature as a result [16]. Its incorporation determines the films' tensile strength, flexibility and even adhesive properties [1,75]. The addition of a plasticiser also decreases the viscosity of the polymer solution, since it reduces the intermolecular forces between the polymer chains and improves mechanical strength (Fig. 5) [7]. Plasticisers can also increase the mobility of polymer chains and free up volume in the structure of the film, thereby improving drug release [16].

When choosing the plasticiser, its compatibility and miscibility in the matrix-forming ingredient solution must be taken into account. The ideal plasticiser must be able to reduce the glass transition temperature without negatively affecting the film's mechanical properties or permeability. In this search for the ideal plasticiser, films are usually prepared from combinations of two or more plasticisers. The most frequent method is to add the plasticiser before the formation of the film, incorporating it into the polymer solution. However, there is also the possibility – albeit not widely used – of incorporating the plasticiser later, causing it to be absorbed by the film [39].

Polyols are possibly the most commonly used polar plasticisers [85]. Glycerol is the simplest, although other substances can be found with higher molecular weight and more hydroxyl groups. Some authors have noted in hydrophilic polymers that the plasticisation efficiency increases with polyols with lower molecular weight, since more

Table 1
Natural film-forming ingredients for fabrication vaginal films. References to their use in vaginal films are included.

Polymer	Origin	Source	Solubility	Plasticisers	Fabrication comments	References
Pectin	Plants	Heteropolysaccharide from the pericarp of some fruits (apples or citrus)	Moderately soluble in water Practically insoluble in organic solvents	Glycerol Polyethylene glycol 4600	Requires quick drying to prevent microbial contamination	[18]
Tamarind seed polysaccharide	Plants	Polysaccharide from seeds of *Tamarindus indica* L.	Soluble in water	Propylene glycol	Temperature can be increased to 50–80 °C to accelerate drying	[15]
Fenugreek gum	Plants	Polysaccharide from seeds of *Trigonella foenum-graecum*	Soluble in water	Glycerol	Temperature can be increased to 45–50 °C to accelerate drying	[17]
Tragacanth gum	Plants	Polysaccharide from the dry sap of *Astragalus sp.*	Soluble in water Insoluble in organic solvents	Glycerol	Dissolves even in cold water	[41]
Zein	Plants	Protein from corn	Poorly soluble in water Soluble in acetone, acetic acid, aqueous alkaline solutions and in aqueous alcohols	Glycerol Polyethylene glycol 400 Oleic acid Tributyl citrate	Dissolves in 50–95% alcoholic solution or 70–80% acetic acid solution in water Heating not necessary if alcohol concentration is 80–95%	[24]
Gelatin	Animals	Protein obtained by collagen hydrolysis	Soluble in water and hydroalcoholic solutions	Propylene glycol	Add to water, heat to 50–70 °C and maintain for 30 min, cool to 35–40 °C Add plasticiser at the maximum temperature or after cooling	[66]
Chitosan	Animals	Heteropolysaccharide from the exoskeleton of crustaceans, obtained through partial deacetylation of chitin	Low solubility in water at neutral pH	Propylene glycol Polyethylene glycol 400 Lactic acid Tartaric acid Citric acid	Dissolves in aqueous acid solutions This acid can also serve as plasticiser	[43,66–68]
Carrageenan	Algae	Heteropolysaccharide extracted from red seaweed	Soluble in water	Glycerol Polyethylene glycol 400	λ-carrageenan does not have good film-forming properties and must be combined with other polymers. K and ι stand out for their film-forming properties, but have not yet been used for vaginal films.	[69,70]
Alginate	Algae	Polysaccharide extracted from brown seaweed	Soluble in water	Glycerol Propylene glycol Polyethylene glycol 400	Temperature can be increased to 45–50 °C to accelerate drying Film characteristic affected by pH and metal ion concentration	[71,72]
Xanthan gum	Bacteria	Polysaccharide produced by *Xanthomonas campestris*	Highly soluble in water	Glycerol	Temperature can be increased to 40–50 °C to accelerate drying	[41,73]
Gellan gum	Bacteria	Polysaccharide produced by *Pseudomonas elodea*	Soluble in water	Propylene glycol	Temperature can be increased to 40–60 °C to accelerate dissolution and drying	[7]
Pullulan	Fungi	Polysaccharide produced by *Aureobasidium pullulans*	Soluble in water	Polyethylene glycol 4000	Can be fabricated at room temperature	[74]

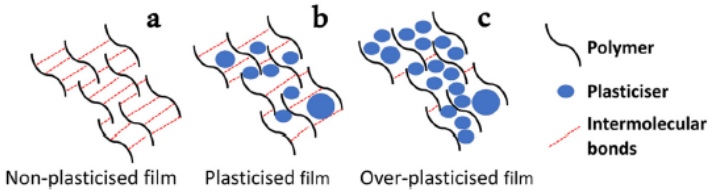

Fig. 5. Interactions in a non-plasticised film (a), a plasticised film (b) and an over-plasticised film (c).

plasticiser molecules come into contact with the polymer chains [86,87]. The addition of these molecules is especially relevant when the films are prepared from an aqueous solution, as their ability to improve their mechanical properties is based primarily on their capacity to increase the moisture retained by the polymer [88]. Glycerol is without doubt the most frequently used polyol in film fabrication. It is a small molecule that can be incorporated into both hydrophilic and amphiphilic polymers [20,35], and it is widely used as it is economical, biodegradable and very resistant to high temperatures [34]. Another polyol commonly used in films is polyethylene glycol, which is larger than glycerol, but has the advantage of having a wide variety of molecular weights available, making it possible to select the one that best suits the characteristics of the polymers [8]. Although less common, there are also films that are plasticized with other types of polyols, such as propylene glycol, triethylene glycol, sorbitol or mannitol [13,39].

Organic acids are another group of hydrophilic substances that have been used in plasticizing films. As in the case of polyols, the molecular size of these acids is a variable factor that can be considered depending on the mechanical properties desired in the films. The three most frequently used organic acids for this purpose, ordered from smallest to largest, are lactic acid, tartaric acid and citric acid [43,80,89,90], all of which are characterised by having at least one acid group and one free hydroxyl group, so they can form hydrogen bonds with hydrophilic polymers [91]. When the aim is to prepare films based on polymers and which require an acidic pH to dissolve (such as chitosan or carrageenan), a solution of these organic acids is usually prepared to serve as a vehicle and also act as a plasticiser [67,92].

It should always be borne in mind that water is generally an excellent plasticiser for films, so the higher the residual water content in the structure, the better the mechanical properties of the film. Many polar plasticisers produce an increase in the film's residual water retention. Several polar plasticisers are often combined, since it is preferable to use small amounts of several plasticisers rather than higher amounts of a single one [81]. This combination can also give rise to a synergistic effect that achieves better plasticizing efficiency with a lower total amount of plasticiser [84].

Although not as frequent as water-soluble plasticisers, non-water-soluble plasticisers have also been used. They may offer slower release of the incorporated drugs, since they make the film more waterproof to aqueous media [75]. One group of amphiphilic substances that can be used as film plasticisers are fatty acids (mainly oleic acid), which are capable of modifying their arrangement depending on the polymer used; so in the presence of a hydrophilic substance, the carboxylic head interacts with the polymer, leaving the non-polar tail exposed to the outside, and when it comes into contact with a hydrophobic surface, the polar head is oriented outwards [93]. Finally, another group of plasticisers comprises the esters obtained from the esterification of organic acids, which transforms them into molecules with a predominantly non-polar character (although they still have some free hydroxyl group). The most frequent are citrates (triethyl citrate, tributyl citrate). They are used practically exclusively in films formulated with polymers with a marked hydrophobic character, such as ethyl cellulose, polymethacrylates or zein [24,41,43].

3.3. Film fabrication

The most common technique for fabricating vaginal films is the solvent casting method [15,19,81], which involves preparing a polymer solution, which is subsequently poured onto a substrate (which may be aluminium [94], glass [21,58,75], Teflon [9,35,95], polyethylene [20,34], plastic [29,36,96] or silicone [7,24]). Individual templates can be used to obtain films with the final size [24,80] directly, or a template that produces a large film that is then cut into several films with the final size [18,38,97]. In this second option, it is essential to guarantee the homogeneity of the film. In some cases, if the solution has high viscosity and many bubbles accumulate in it, the solution can be sonicated to remove them [10,98]; while in others, the pH of the solvent must be adjusted to ensure the complete dissolution of the polymer [58]. Finally, the solvent is removed by evaporation, causing the polymer molecules to reorient and intertwine with each other, forming a film (Fig. 6) [7,94]. When films are prepared containing two or more polymers with very different solubility, the most frequent procedure is to use a mixture of several solvents to achieve the dissolution of both polymers.

This is a versatile technique that can be applied in both the laboratory and at the industrial scale. At the industrial level, automatic film applicators produce films by solvent casting, so they can be easily and continuously fabricated from a polymer solution, without the need

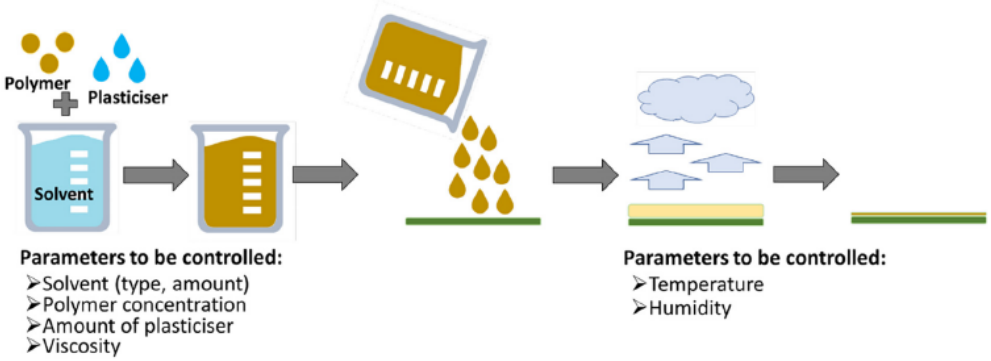

Fig. 6. Diagram of film fabrication by the solvent casting method.

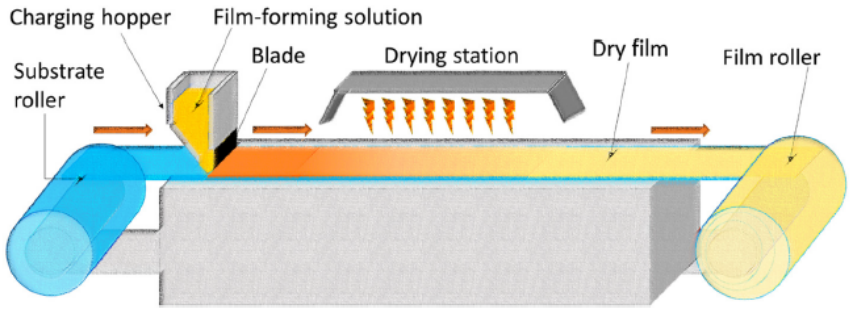

Fig. 7. Diagram of a system for continuous film fabrication by solvent casting.

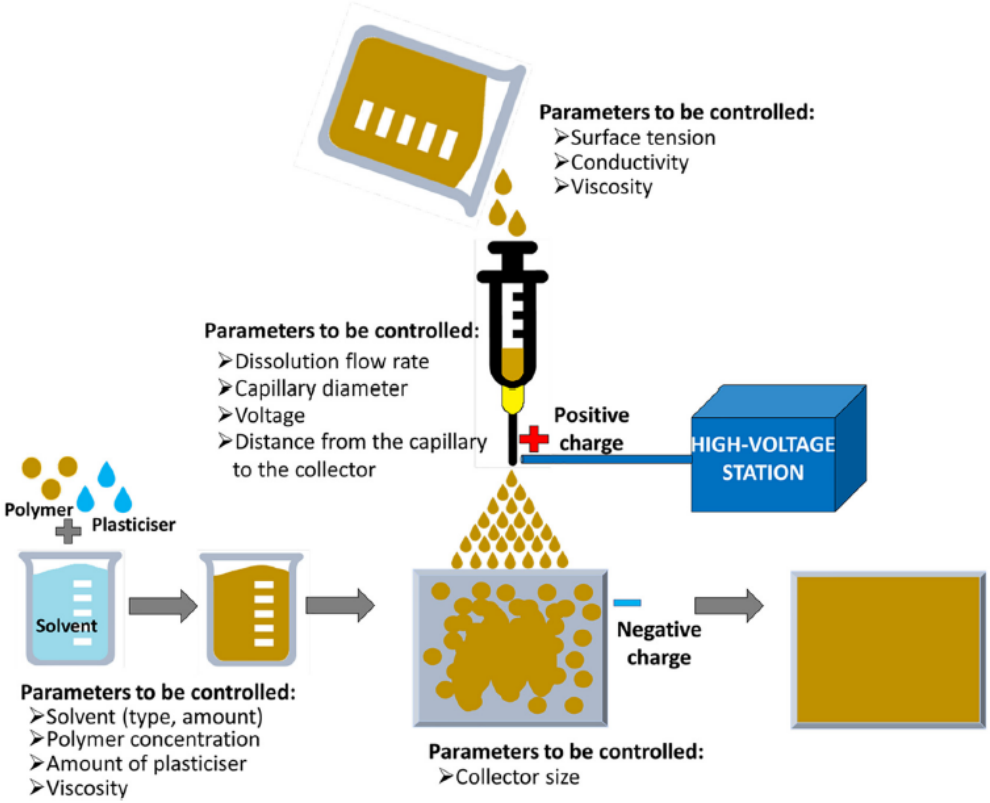

Fig. 8. Diagram of film fabrication by electrospraying.

for additional templates or drying equipment [13,74]. This equipment makes it possible to control the drying temperature and the film thickness (Fig. 7) [99].

Some researchers have included variations to this technique: drop-deposition is a procedure whereby the solution is added dropwise to the template, while spin casting is when the mould with the polymer solution is spun [96]. However, neither of these techniques appear to offer any improvement in film fabrication and have very little application at the industrial level.

Despite the simplicity of this technique, there are many variables that must be controlled due to their ability to significantly modify the films' properties. The concentration of the polymer in the dissolutions has the greatest influence on the films' characteristics [15]. The thickness of the film is proportional to the amount of polymer, and conditions both the mechanical properties and the rate of release of the drug [7]. When the films come from very concentrated solutions, larger aggregates of particles are formed, and the molecular interactions are consequently weaker [39]. It is very frequent to find a "limit" of plasticity when increasing the concentration of plasticiser in the film; that is, there is a point after which the addition of more plasticiser produces an anti-plasticizing effect. This effect has been associated with the fact that an excess of plasticiser causes phase separation and excludes the plasticiser from the film structure, possibly damaging the continuity of the polymer network and rendering the resulting film more fragile [67].

The solvent is also crucial. Water, alcohols and various organic solvents and their mixtures can be used to prepare polymer dissolutions or suspensions (hydroalcoholic mixtures are quite frequent [94]) that allow better control of the evaporation rate, which is a clearly determining factor in the film structure [36]. When films are prepared from a suspension, its viscosity can influence the distribution of the drug in the film; high viscosity may result in an uneven distribution of the active ingredient.

Finally, environmental conditions (temperature and humidity) clearly affect evaporation, and are also a determining factor. Examples

can be found where the film solution evaporates at room temperature [94,95], and others where the temperature increases slightly to 35–45 °C [19,35]. It should always be considered that slower drying will produce more homogeneous films [34], although if water is used as a solvent, excessive time until evaporation could lead to microbial proliferation in the solution, which would require the incorporation of preservatives to avoid it. There are also more extreme cases in which higher temperatures (up to 120 °C) are used in order to considerably reduce the films' fabrication time [20,75,100]. It is necessary to take into account the stability of the plasticisers, film-forming substances and active ingredients, so it is often inviable to raise the temperature very high due to degradation issues [10]. The humidity parameter is also often controlled at the industrial scale. In general, a relative humidity of 50% at 25 °C is considered adequate to achieve an optimal evaporation rate.

Nevertheless, it should also be noted that alternative methods to solvent casting have been explored, although they have as yet barely been applied in the fabrication of vaginal films. Electrospinning and electrospraying are two examples. The basis of these techniques is the application of an electric field on a capillary – through which a polymer solution flows – and a collector. As this electric current accelerates, the polymer molecules move to the collector while the solvent evaporates (Fig. 8) [101–104]. The polymer concentration in the solution is crucial; drops are obtained at low concentrations (electrospraying), while fibres are formed at higher concentrations (electrospinning). In electrospraying, films are formed by accumulating numerous droplets on the collector, and in electrospinning, fibres with a vast specific surface can be grouped to obtain nanofibre-based films, although the systems obtained by this technique are not considered films by some researchers, since they are not continuous matrices [105]. In addition to the properties of the polymer solution, several technical parameters must also be managed in these techniques (capillary diameter, voltage, flow rate, distance to collector and its size, *etc.*). These techniques could be easily scaled-up to industrial level. Their main inconvenience may be the higher production time and costs, compared to solvent casting.

Another alternative – again not yet widely applied for vaginal films so far – is compression moulding or extrusion. The compression moulding technique consists of melting polymers or their mixtures together with plasticisers and active ingredients to obtain a granulate. The granules are melted in a compression moulding machine, and this mixture is then compressed between steel plates and rapidly cooled to obtain films (Fig. 9) [106,107].

In a similar process, vaginal films have also been fabricated by hot melt extrusion (Fig. 10) [108], in which the main parameter to be controlled is the temperature. High temperatures (120–240 °C) are usually attained, but this depends on the melting temperature or the glass transition temperature of the mixture [106,107,109]. This is an important limitation of this technique, since only heat-resistant materials and active ingredients can be used. The pressure and compression time and the screw speed when the mixture is extruded also require control.

Finally, the newest method used to obtain films is 3D printing. The most common technique is fused deposition modelling, in which a strand of thermoplastic polymer is pressed by rollers inside a nozzle, which applies heat to melt the polymer, which is then deposited on a suitable surface to solidify [110]. The film is prepared layer-by-layer, making it possible to create sheets with the desired geometry and exert closer control over the disintegration of the film and the drug release. Films created by 3D printing are a more personalized way to create films, although the cost and production time is markedly higher than in more traditional techniques, so their applicability at the industrial scale is extremely limited [111].

3.4. Drug loading

The way the drug is incorporated into the films is also important. Obviously, the film must allow the incorporation of the complete dose of the drug. It should be considered that the addition of an active substance implies the presence of foreign particles in the polymer structure which, if excessive, could lead to a heterogeneous film and even jeopardize its continuity.

The most frequent strategy is undoubtedly the dissolution of the active substance in the polymer solution, so that once the solvent has evaporated, the drug is integrated in the polymer structure of the film [7,10,20,95]. This can only occur if the drug is soluble in the solvent used with the polymers. It is sometimes necessary to dissolve the drug in a different solvent to the one used to prepare the polymer solution and form the film matrix (when the drug is not soluble in this solvent), and the two solutions are then mixed just before casting [13].

Another option available when the drug is not soluble in the medium in which the films are prepared is to incorporate it as a dispersed suspension in the polymer solution [17]. In this case, a small amount of surfactant is sometimes added to the solution to promote the dispersion of the drug [15,92]. Irrespective of how the drugs are incorporated, *i.e.* dissolved or dispersed, the final films must be homogeneous and have no hard edges or spots that could cause trauma when used, or even offer poor organoleptic properties (*e.g.* "dusty" films or heterogeneous inclusions of drug in the film matrix).

Films can also be used as a vehicle to incorporate drug-loaded nanoparticles. These films are produced by incorporating the active ingredient into the nanoparticles and then preparing a dispersion in the same solvent used to obtain the film. The film's component polymer solution (and plasticisers, if any) is prepared, and once dissolved, the nanoparticle suspension is added on this polymer solution [29]. A single solution of the film components can also be prepared directly, in which the nanoparticles are dispersed just before casting [12,38]. The resulting mixture is used to obtain films, usually by the solvent casting method. The incorporation of the drug in nanoparticles is particularly interesting in the case of drugs with different solubility profiles [19]. However, it should be noted that one possible drawback is the

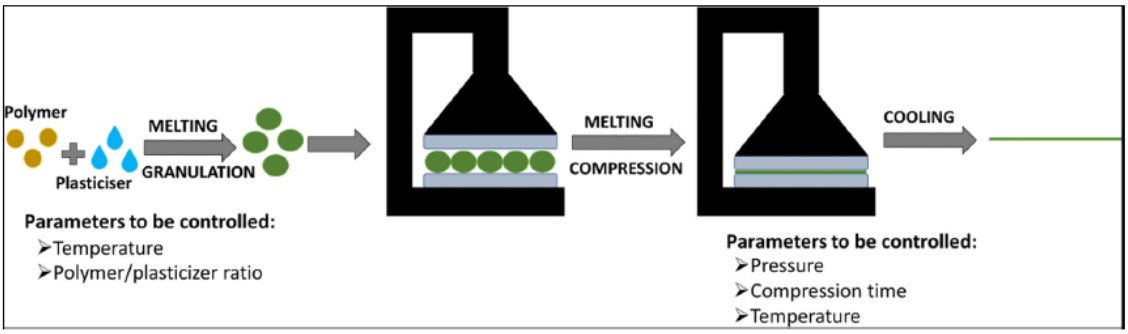

Fig. 9. Diagram of film fabrication by compression moulding.

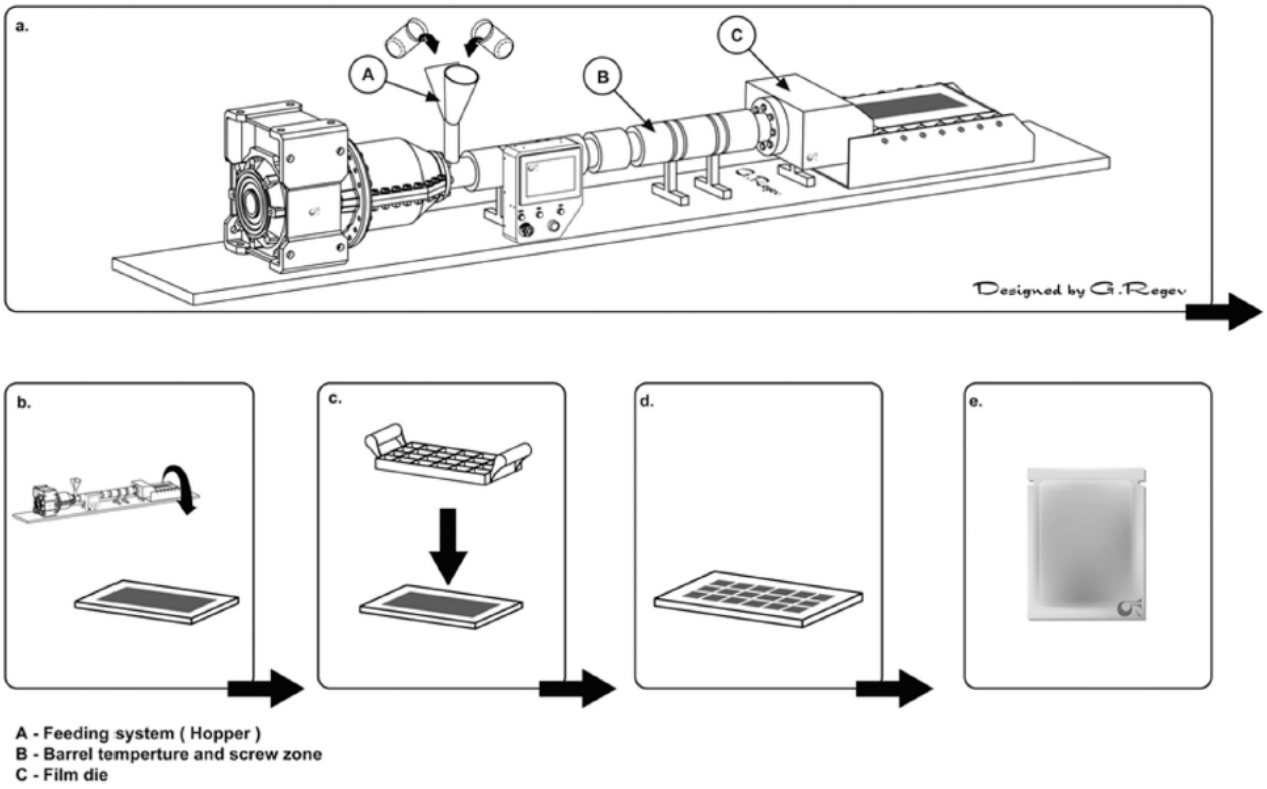

Fig. 10. Schematic diagram of the hot melt extrusion process for vaginal film manufacturing. Film generated from the extruder system (a) is then transferred to the cutting stage (b) and a cutting die (c) is used to generate individual unit doses (d) which are then packaged into foil pouches (e). Reprinted by permission of Springer Nature from [108], Copyright (2019).

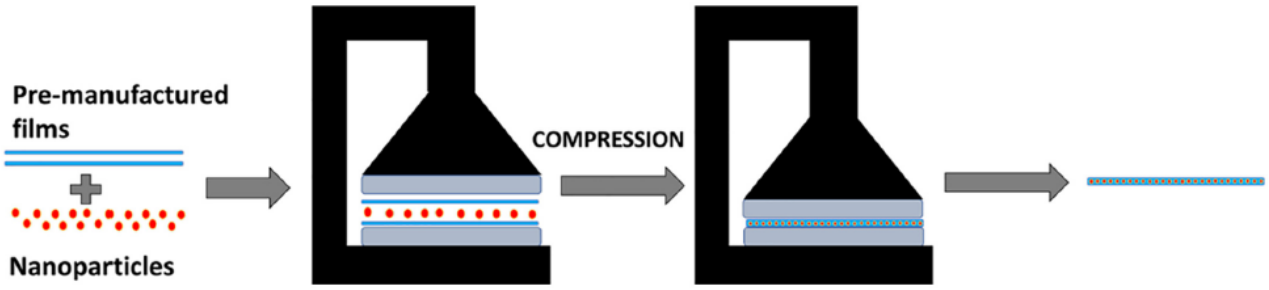

Fig. 11. Diagram of the incorporation of nanoparticles between two films to obtain layer-by-layer drug-loaded films.

premature release of the drug from the nanoparticles into the solvent in which the films are prepared during the drying step of the solvent casting. Non-solvents of the drug can be used to avoid this. However, when this is not possible, premature drug release may be minimised by shortening the drying time of the films (*e.g.* by raising the temperature, decreasing the pressure, increasing the air renewal flow, reducing the amount of solvent, *etc.*) [4]. The incorporation of nanoparticles in bi-layer films has also been proposed as an alternative. What is innovative is that the drug-loaded nanoparticles have been incorporated between two pre-formed film layers that are subsequently compressed together using a hydraulic press (Fig. 11) [18]. Although this is feasible at the laboratory scale, its transition to industrial fabrication may pose a challenge.

4. Characterisation

Once the films have been prepared, they must be characterised to assess both the uniformity of the fabrication method and their properties. According to studies by the various research groups working on the development of vaginal films, the main characterisation studies that can be performed are described below.

4.1. Visual assessment

The first analysis that is usually done on the prepared films is a visual evaluation to determine their colour, transparency, uniformity, surface (smooth or porous), and presence or absence of cracks and flexibility (Fig. 12) [9,19,95]. In some cases beads are observed in the film, which may be due to the nature of the components [94] or a sign of an error in the formulation. A key factor explaining the absence of macropores seems to be that intra-molecular aggregates do not occur between the polymer chains, allowing the solvent to evaporate easily [112]. The films' appearance will have a major impact on user adherence. They must be smooth, thin, and preferably translucent to be

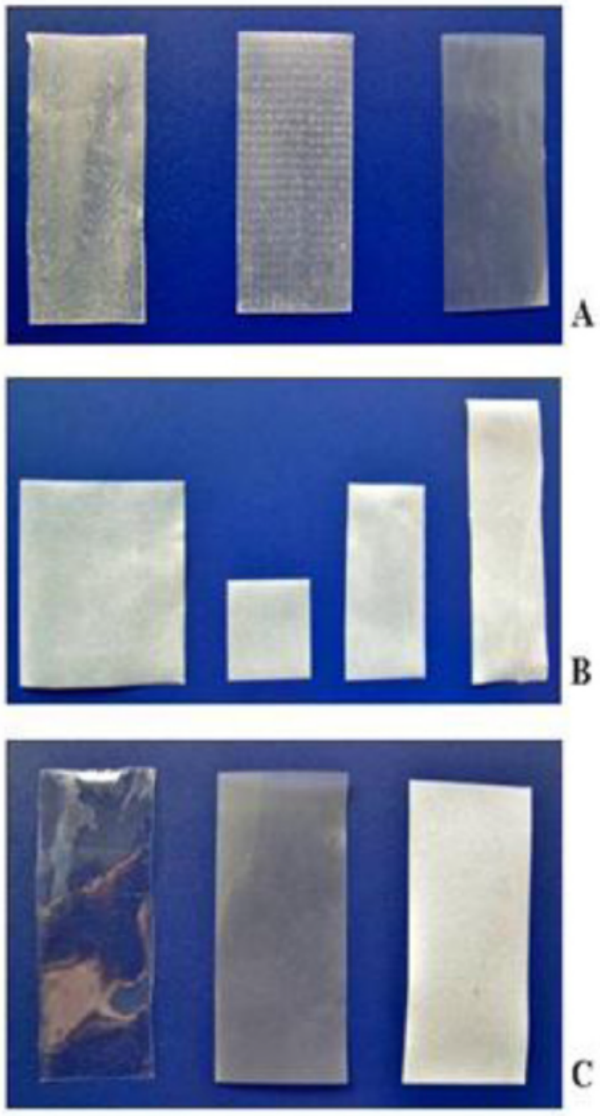

Fig. 12. Demonstration of vaginal film products. (A) Films of varying texture and thickness. From left to right: textured/thin, textured/thick, smooth/thin. (B) Films of varying size and shape. From left to right: 5 × 5 cm, 2.5 × 2.5 cm, 2.5 × 5 cm, 2.5 × 7.6 cm. (C) Films of varying appearance. From left to right: clear, translucent, and opaque. Adapted by permission of Springer Nature from [28], Copyright (2016).

widely accepted by women [2].

4.2. Uniformity of the films' mass and thickness

The assessment of the uniformity of the films' mass – by individual weighing – is important to guarantee the accurate dosage of individual units [15,17]. Another simple characterisation of films' uniformity is the determination of their thickness, for instance, using a micrometer [9,20,21,35].

4.3. Characterisation of hydrophilicity/hydrophobicity and related properties

An assessment of the water uptake of the formulations gives an idea of how the films behave in the presence of vaginal fluid after administration. The swelling test is especially useful in the case of polymers capable of gelling. This is done by immersing the films in water or simulated vaginal fluid under physiological conditions (pH 4.2, 37 °C) and weighing them at different time intervals to determine their swelling profile [7,67]. This test can be prolonged over time, and in biodegradable films, the weight loss due to disintegration or erosion will also be observed [24]. The water uptake or swelling ratio (*SR*) can be calculated as the percentage of weight gained compared to the initial weight [8,27].

A variation of this test consists of determining the resistance to dissolution/disintegration in a given solvent. The film is immersed in this solvent and extracted, dried and weighed after a certain time to evaluate the weight loss [8,20,34,37]. It can also be examined by scanning electron microscopy (SEM) to observe how its structure has been modified [94]. Some researchers evaluate the disintegration time by visually evaluating the film until it is observed to have completely disintegrated [18,38,82]. When water capture significantly modifies the film's dimensions, the swelling index (*SI*) can also be evaluated, not as a modification of the weight but of the diameter of the films [15,17].

One research group has also analysed films' ability to capture water using the quantitative imaging refractometry method, in which a two-dimensional structural hydration and disintegration model is designed based on the analysis of changes in volume and density in the presence of a fluid (Fig. 13) [23].

Although the films' swelling ability will depend mainly on the polymers in the formulation, these tests also evaluate the influence of plasticisers on the films' ability to capture water [66]. The inclusion of plasticisers modifies the structure of the films, and swelling can be affected as a result.

Another parameter that reveals the hydrophilicity/hydrophobia of the film's surface and the initial degree of wetting of the film is the water contact angle (θ) [8,37]. This can be determined using the sessile drop method (Fig. 14) [15,20,93,94,113]. A small contact angle implies a strong interaction between the water and the surface of the film, while if the contact angle is higher it indicates that the attraction between the surfaces is weak, so the film is more hydrophobic [34,95]. The main limitation of this technique is its lack of application to fast-dissolving films, since the water drop is immediately absorbed in the film. Some authors have also stated that the contact angle may be related to the mucoadhesiveness of hydrophilic films, but only up to a point, after which greater water uptake detracts from the adhesiveness and causes a leakage in the cohesiveness of the film, while reducing the adhesion force due to disentanglement at the interface between mucosa and film [8]. The adhesion work (W_a) and the spreading coefficient (*SC*) [17] can be calculated from the contact angle value.

The moisture content of the films has also often been characterised. This can be determined using a heating balance [18,82,114] or by weighing the films and subsequently placing them inside a desiccator with calcium chloride at 40 °C for 24 h [27]. However, the most robust method for determining residual water content is the Karl Fischer titration, based on a reagent that converts water into a non-conductive substance [115].

One methodology that has been used to evaluate the hydrophilicity of films is the study of the sorption isotherms. Using a DVS Intrinsic apparatus, the samples are exposed to different relative humidity values at room temperature [37]. The comparison of the values obtained in different films gives an idea of their ability to capture water, which is related to their hydrophilicity [21].

Finally, the determination of the pH of films after dissolution or dispersion is important when assessing possible irritability in the vaginal mucosa while the film remains attached. It must be ensured that the pH and osmolarity conditions of the vaginal fluid are not significantly affected by the presence of the film [17,38].

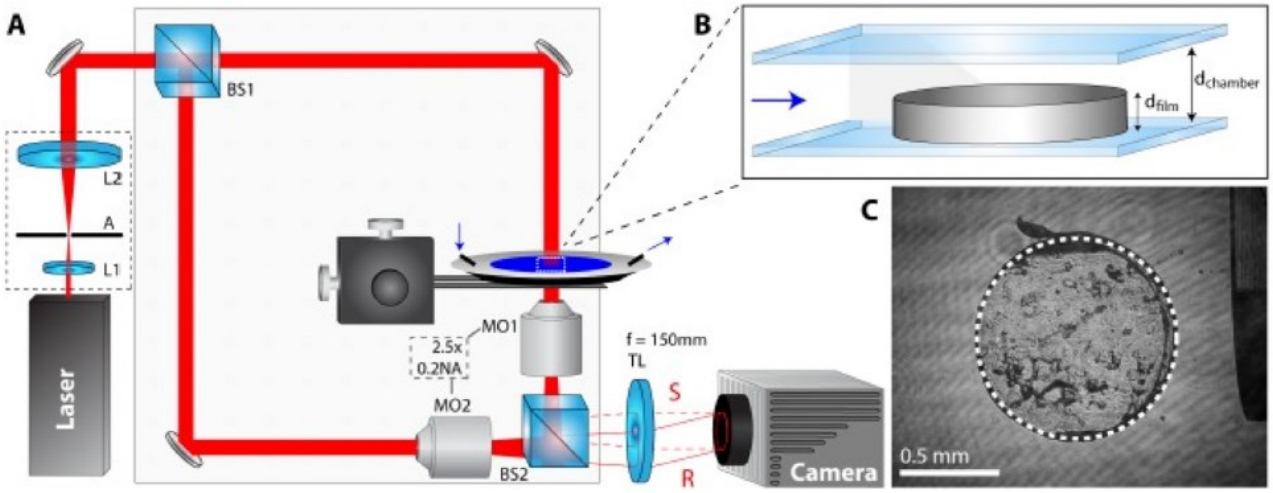

Fig. 13. Microscope setup and hydration assay configuration. (A) Experimental schematic of quantitative phase microscope. (B) Film geometry within the chamber at time t = 0. The blue arrow indicates lateral water flow which initiates hydration. (C) Typical interferogram of a 1-mm-diameter film sample with the phase calibration ramp visible in the microscope's field of view (right edge). Reprinted from [23] under the terms of the Creative Commons Attribution License 4.0 (Copyright 2014 Rinehart et al.; doi: https://doi.org/10.1371/journal.pone.0095005). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

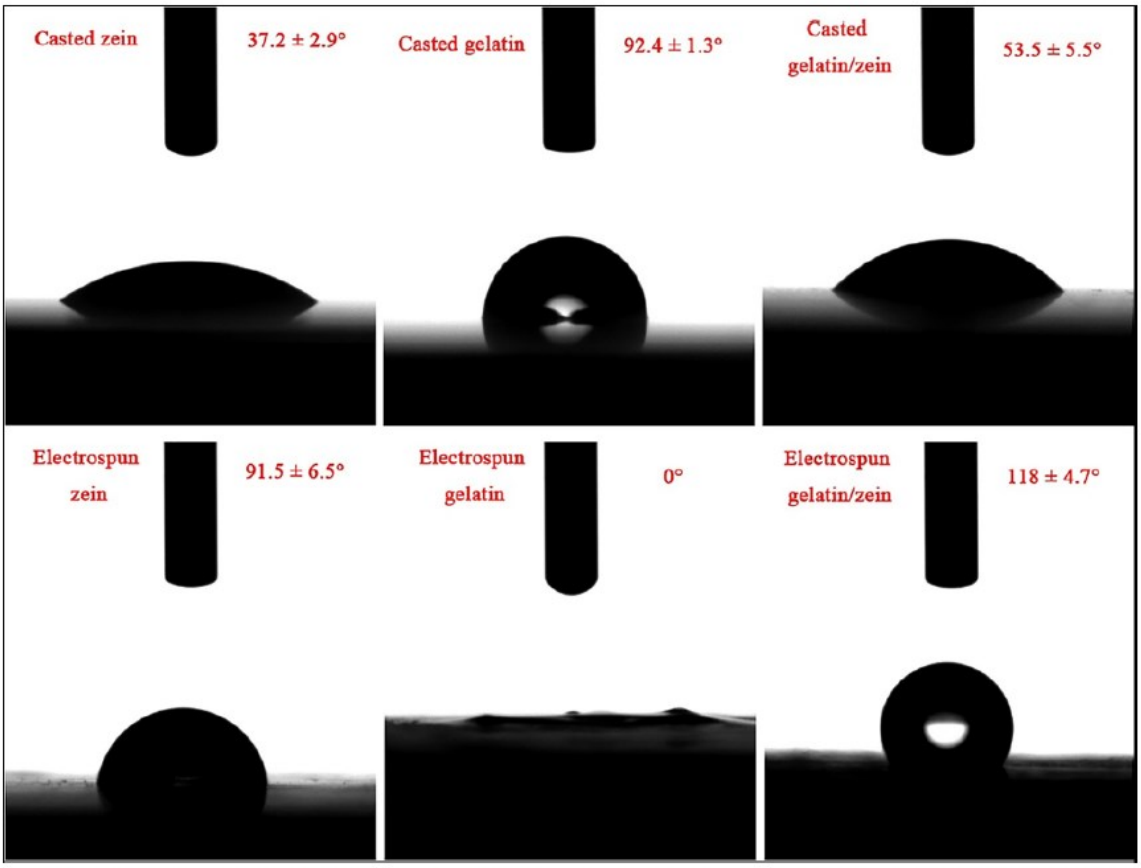

Fig. 14. Water contact angle measurements of the electrospun and casted gelatin, zein, and gelatin/zein (1:1, *w*/w) films. Reprinted from [94], with permission from Elsevier, Copyright (2017).

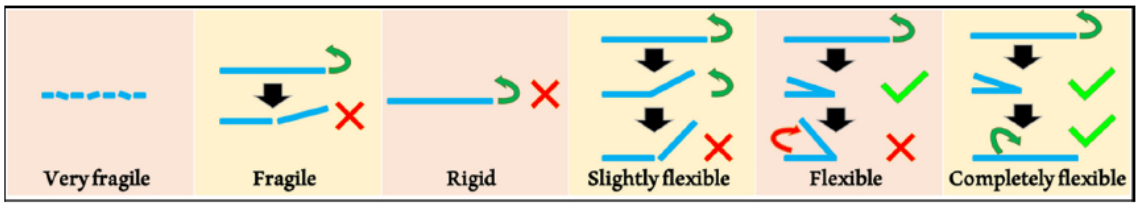

Fig. 15. Graphic representation of the film pliability classification proposed by Notario-Pérez [24].

4.4. Mechanical properties

The films' mechanical properties are often initially evaluated by determining their folding endurance. The films are manually folded over the same point until they are broken, or until a limit of folds is reached [15,17,19,67]. Other researchers conduct a qualitative in-hand inspection of the pliability of the films. Ham et al. classified the films as possessing high or low pliability by folding them on a 1 cm roll [82]. Notario-Pérez et al. have established a more detailed classification of the pliability of films (Fig. 15). The film is folded in half and released to allow it to recover its initial form. Based on how they behave, films can be classified from very fragile to completely flexible [24].

From a quantitative point of view, the assay that has become essential for characterising films' mechanical properties is the evaluation of flexibility and fracture resistance. For the vaginal application of films, and to adapt to the movements of the body's mucosal surface, they must have sufficient resistance to withstand any tension they may undergo during application [8,17,38]. These properties will depend mainly on the polymers forming the film, but can be modified with the incorporation of plasticisers [67], and when the film contains nanoparticles [29].

The elongation test consists of determining tensile strength and elongation until breaking, and these measures can be obtained with a texture analyser [21,97] or a universal testing machine [27,36] (Fig. 16). The elastic modulus can also be calculated from the data obtained in this test [8,34,67,113,114]. A decrease in elongation to the breakage of films indicates greater stiffness [95]. Another option is to use the texture analyser to perform puncture tests (Fig. 16) [18,19,24]. The puncture strength, elastic modulus and burst distance can be determined by this method [73,99].

4.5. Imaging techniques

The morphology and homogeneity of films is often evaluated in a simple way using scanning electron microscopy (SEM) [29,35,58,94] to visualize both their surface structure and their inner structure by observing a cross-section of the film (Fig. 17) [21,97,114]. Some researchers also use SEM to approximate their thickness, but it should always be noted that it may be slightly reduced due to the vacuum created in the microscope chamber [19]. The presence of any particles and imperfections on the surface of the films can easily be seen with this technique [29]. In some cases, small bubbles have been observed inside the films that cause an increase in their surface area, leading to greater fluid uptake and faster drug release [7].

In the case of blend films, a smoother and more homogeneous structure than in films containing the same polymers separately is usually indicative of good miscibility between polymers and the homogeneity of the mixture [36,58]. This technique can also be used to confirm the formation of multilayer films [43]. Films incorporating nanoparticles usually have a more compact and smoother structure with fewer surface pores and cracks. It could therefore be assumed that the presence of nanoparticles provides a substrate on which the film will subsequently form, thus making the final structure more robust. However, when an excessive number of nanoparticles is incorporated, the opposite is observed: a greater presence of microcracks that have been attributed to an agglomeration of nanoparticles [29]. This technique also reveals whether the nanoparticles are evenly distributed or if any aggregates have been produced [38]. It is therefore necessary to incorporate an adequate number of nanoparticles to guarantee their homogeneous dispersion.

As an alternative, confocal microscopy allows the observation of the films in their final state, and also the effect of different solvents on their structure (Fig. 18) [94]. This technique can also be used to evaluate the arrangement of the various components in the film's structure [37].

4.6. Structural characterisation

It is essential to determine the residual organic solvent content, since pharmaceutical legislation is quite strict in regard to the content in these substances. Large amounts of organic solvents are usually required to obtain the films – when the films cannot be prepared with water alone –, so the residual content of these substances must be

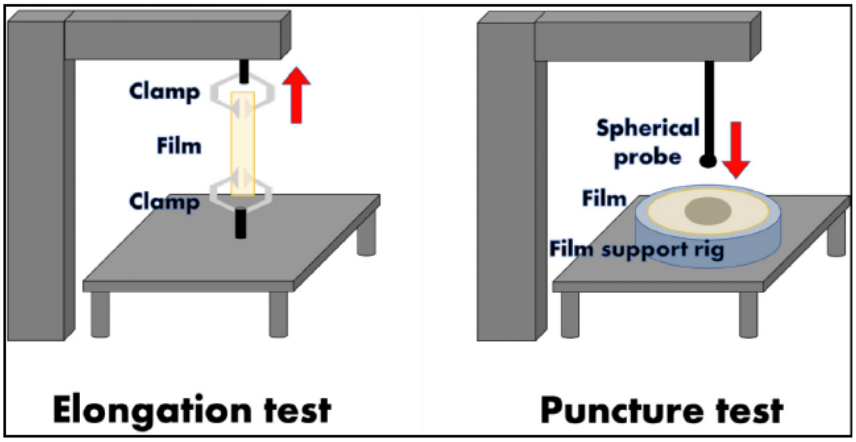

Fig. 16. Graphic representation of the elongation test (left) and puncture test (right) to characterise the films' mechanical properties.

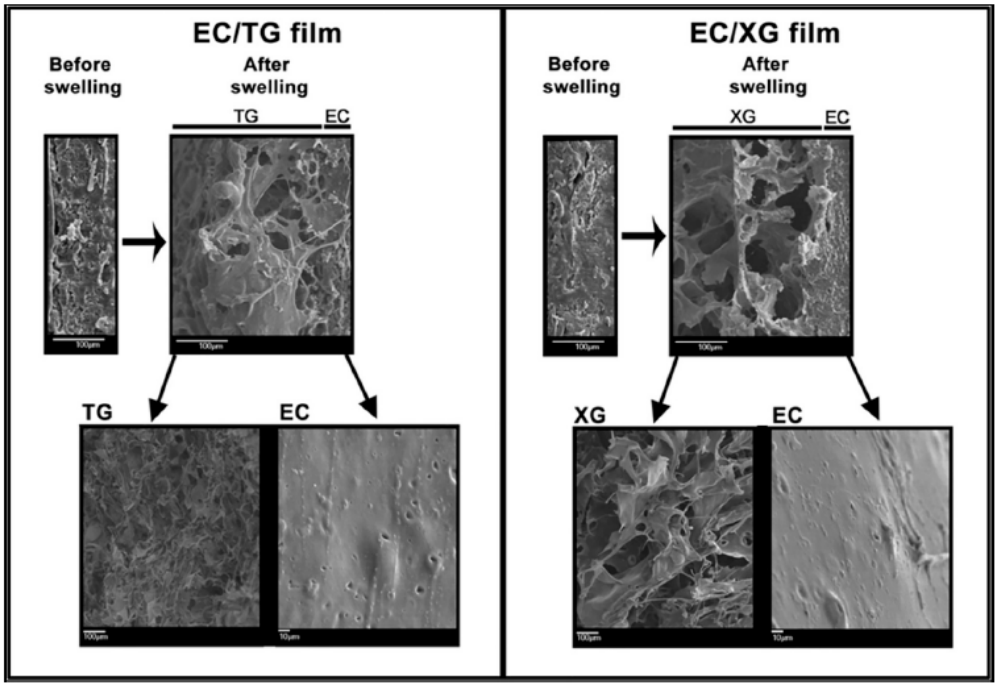

Fig. 17. Images obtained by SEM of films containing ethylcellulose/tragacanth gum (EC/TG; left) and ethylcellulose/xanthan gum (EC/XG; right). The cross section of the films before and after swelling is shown above. The surfaces of both faces of the films after swelling are shown below. Reprinted with permission from [41]. Copyright (2020) American Chemical Society.

determined at some point prior to their evaluation in clinical trials. It is most frequently quantified by gas chromatography, although thermogravimetry can also be used [9,21]. The headspace analysis by gas chromatography is suggested to determine residual solvents in drug products [116]. A method to determine residual organic solvents in films was described by Ozaki et al. and consists in the addition of a solution of *N,N*-dimethyl formamide to the sample. After overnight incubation at room temperature, the headspace gas is analysed by gas chromatography, being possible to detect the presence of at least 30 substances such as acetone, methanol, ethanol or ethyl acetate, which are commonly used for films fabrication [117].

Another technique to determine the films' chemical structure is Fourier transform infrared spectroscopy (FTIR). The infrared absorption spectrum allows the characterisation of the functional groups in the films and the identification of structural changes [29,34,97]. The bands observed provide information on the possible interactions between the different film components [100]. This technique can also reveal chemical changes that commonly occur in films due to plasticisation [118]. One observation that is fairly simple to interpret is the formation of hydrogen bonds, which generally leads to an improvement in the film's mechanical properties [21,36,94].

Similarly, X-ray diffraction can be also useful for the films' structural characterisation. The comparison of the films' X-ray diffraction pattern with those of their components separately can provide information on their interactions if this causes a change in crystallinity [29,67,114]. Blend films can also be compared to films obtained from only one matrix-forming ingredient, in order to observe any possible interactions during the film formation [37].

4.7. Thermal studies

Differential scanning calorimetry (DSC) analysis provides knowledge of the films' temperature and enthalpy of fusion [94], glass transition temperature [35,96], and other thermal transitions [34]. These parameters offer valuable information; for example in blend films they reveal the interaction between the components [37]. If the melting temperature or the glass transition temperature of the mixture is lower than that of the polymers separately, this indicates that a phase separation of the polymers takes place, and that the interaction between the polymers is weak. In contrast, a higher melting temperature of films made from a polymer mixture suggests an interaction between the chains of the two polymers due to the formation of new bonds that stabilize the thermal properties of the film [37,94]. Similarly, this technique can be used to assess the compatibility of the polymer with the plasticiser [84].

A similar phenomenon can be observed in the glass transition temperature when the films are amorphous; an increase in the glass transition temperature will imply a more robust and orderly structure of the polymers in the film, while a lower glass transition temperature may be related to a more flexible structure [35]. This occurs through the addition of plasticizing substances, which decrease the glass transition and increase flexibility [34,75]. This process is known as plasticisation, which implies a decrease in the T_g of the polymer and an increase in the elastic modulus, producing greater flexibility [84]. However, it should be noted that the opposite phenomenon, known as anti-plasticisation (in which the addition of a substance causes an increase in the T_g of the polymer), can also occur [75].

When the substances in the film melt at the same temperature (which differs from the temperature at which they melt separately), this reveals excellent compatibility between the components, which have been perfectly integrated into the formulation [10].

The determination of thermal stability through thermogravimetric analysis (TGA) is another test that can be performed on films. This is done by exposing the films to high temperatures and observing their degradation and recording their weight loss and energy variations [21]. This test gives an idea of the thermal stability of the films [95], and the comparison of the curve obtained and the curve for the substances separately also provides valuable information about the interaction between the different components [29].

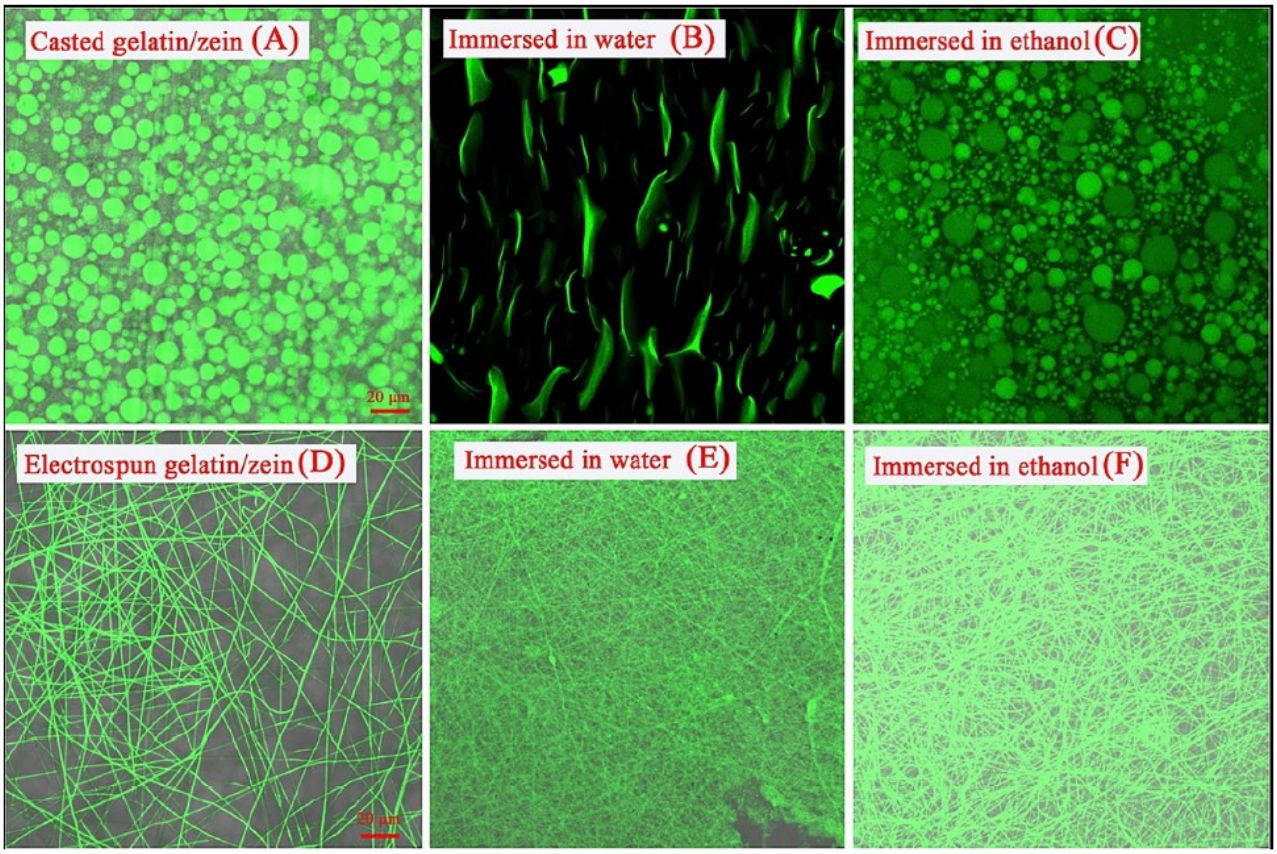

Fig. 18. Confocal microscopic images of the FITC-conjugated casted gelatin/zein film (1:1, *w*/w) before (A) and after immersion in water (B) and ethanol (C) for 24 h; and the electrospun gelatin/zein film before (D) and after immersion in water (E) and ethanol (F) for 24 h. Reprinted from [94], with permission from Elsevier, Copyright (2017).

4.8. Characterisation of drug-loaded films

This drug uniformity test is performed to verify that the drug is incorporated uniformly in the films [7–9,27]. This is very important when films are obtained by cutting a larger film, since the homogeneity of the drug must be guaranteed in all the units obtained from the matrix film. This is done by evaluating the active ingredient content in films obtained from different locations [17].

The release behaviour of the drug from the film is assessed either by immersing the formulation in the release medium (usually simulated vaginal fluid), with an equivalent temperature and shaking to physiological conditions [7,10,18–20,24,67]; or through the dynamic vaginal system, a test some researchers carry out by passing a continuous flow of fluid over the film [8,17,119].

The drug release profile is affected by different factors such as the thickness of the film, its hydrophilicity/hydrophobicity, its morphology and microstructure (presence or absence of micropores), the homogeneity of the drug distribution and the method of incorporation (since it can be encapsulated in particles included in the film, or directly dispersed in the polymer matrix) [39].

4.9. Adhesive properties

The *ex vivo* mucoadhesion test evaluates both the time and strength of the films' mucoadhesion using samples of mammalian vaginal mucosa, usually pigs, cows or sheep [7,10,19,27,67].

The mucoadhesion time study consists of fixing the film to the mucosa by constant pressure and then submerging it in simulated vaginal fluid or dropping a constant flow on it [8,10,24]. The time elapsed until the film detaches from the mucosa is established as the mucoadhesion time (Fig. 19). Another option is the dynamic vaginal system test, which evaluates the volume of film fragments that detach from the mucosa under a continuous flow [7].

Films generally have better mucoadhesive properties than other pharmaceutical forms, due to their high contact surface with the mucosa and their low weight. A direct relationship has also been described between water retention and mucoadhesion time. However, mucoadhesion will depend mainly on the mechanism producing it. Thus adhesion by ionic bonds is usually time-dependent, and generally less durable than hydrogen bonds [7].

Mucoadhesion strength can be determined using a texture analyser (Fig. 19) [17,19,27,38,67], which quantifies the force and detachment work required to detach the film from the mucosa. Some researchers have reported a relationship between the films' mucoadhesion force and their swelling capacity; excessive swelling is generally related to a low mucoadhesion force, because the overhydration of the film causes a disentanglement in the tissue/film interface [17,67].

4.10. Film stability

Although stability studies are essential before any medicine can be marketed, this study is especially useful during the development phase when the drugs in the formulation are easily degradable. The films are stored in suitable packaging (generally aluminium) [10,17]. Following the ICH guidelines, vaginal films are generally stored in intermediate conditions – 25 °C and 60% relative humidity – [19], although

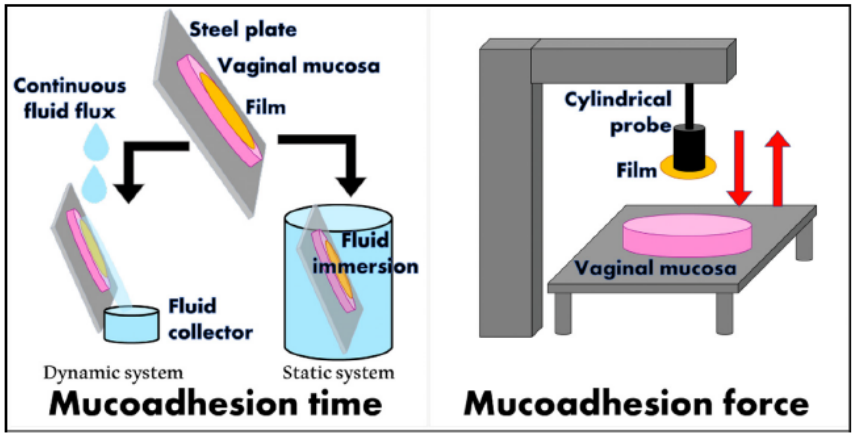

Fig. 19. Graphic representation of the mucoadhesion residence time test (left) and the mucoadhesion force test (right).

accelerated stability can also be studied – 6 months at 40 °C and 75% relative humidity – [9,74]. At several timepoints – 1, 3, 6 and 12 months – the films are removed from primary packages and characterised as already described. The stability test usually evaluates the amount of drug that remains undegraded, as well as changes in weight, thickness, appearance and moisture [13,99].

4.11. In vitro cell studies

The cytotoxicity test is important for understanding the impact of the film formulations on genital tract cells. Common cell lines used in these studies include HeLa cervical, CasKi cervical, HEC-1-A endometrial or VK2/E6E7 vaginal epithelial cells [18,38,74]. Cells are typically incubated with the films for different amounts of time (usually 24–48 h) [17,27] or with a previously prepared film extract [18,38]. Cytotoxicity can be evaluated using standard colorimetric tests of metabolic activity (for example, assays testing the mitochondrial reduction of tetrazolium or resazurin) or leakage (due to cell membrane damage) of intracellular enzymes such as lactate dehydrogenase [7,9,18,67]. Some of these cell lines can also produce and release chemokines/cytokines that provide relevant information on pro-inflammatory responses induced by the drug, excipients and film formulations.

Another interesting preclinical study consists of evaluating the membrane permeability of the drug in the films. This study can be done using epithelial cell monolayer models or more complex cell constructs (lab-made models or commercial versions such as Epivaginal™ from MAtTek or HVE™ from EpiSkin), and animal or human mucosa explants [9,13,120]. These models can be useful for testing drug retention or permeability across the cell barrier, and potential genital toxicity [74,121].

The incorporation of active drugs into films can affect their intrinsic activity due to changes in their release kinetics or even molecular changes, mainly during manufacturing. For example, the activity of antifungal (anti-*Candida*) films can also be easily evaluated *in vitro*. Several authors use agar-based methods to determine the inhibition zone with *Candida* species. The most commonly used test is the agar diffusion method or halo zone test [15,17,67,122,123]. Drug diffusion through the agar and inhibition halos can be measured and related with antifungal activity (Fig. 20). The Clinical and Laboratory Standards Institute (CLSI) reference M44-A2 method or any other standard methodology should be preferably used for agar-based methods in order to sustain higher clinical relevance [9]. Another alternative method to the halo zone test is the reference M27-A4 micromethod from CLSI. Briefly, a *Candida* spp. suspension incubated with a twofold dilution series of film extracts can be used to determine minimum inhibitory concentration (MIC) values. For films containing antivirals, particularly anti-HIV compounds, the inhibitory activity of the drug-loaded film can also be quantified *in vitro* by various cell methods. For example, peripheral blood mononuclear cells (PBMCs) or the TZM-bl cell line can be incubated with films or extracts before being challenged with HIV-1 and further incubated for different amounts of time. The films' inhibitory activity is then determined by monitoring viral replication (when using PBMCs) or by Tat-regulated LTR-driven expression of the luciferase reporter using a luminescence assay (in the case of TZM-bl cells) [9,74,99].

It can also be useful to study the bacterial growth inhibition of films [74,99], by testing either activity against pathogenic bacteria (for example, species of Prevotella, Bacteroides and Mobiluncus, among others) or toxicity against microbiota, particularly lactobacilli. These last are particularly relevant to determine the potential onset of detrimental effects in the vaginal environment [9]. MIC values are usually determined using standardized testing, although one particular alternative test for films includes the determination of inhibitory halos after placing a piece of the film on solid media containing relevant microbiota [27].

4.12. In vivo studies

In order to transfer the research to *in vivo* studies, female mice [19], rabbits [9] or macaques [124] are often used to evaluate the vaginal films developed. Films are particularly important since they can be typically sized to the size of the vagina of the animal used in the experiments, without significantly changing the technological performance of the system. Relevant data such as pharmacokinetics, efficacy or toxicity/safety can be determined depending on the animal model considered and the intended purpose of the films to be tested (Fig. 21) [17,19,38,125]. The animal species should be selected on a case-by-case basis. From a more technical point of view, films are easy to administer (even to conscious animals with proper restraints) and comfortable for the animals once placed in the vagina. However, care should be taken to avoid the expulsion of films due to natural body movements or grooming.

5. Applications of drug-loaded vaginal films

5.1. Prevention of HIV

Vaginal microbicides are topical formulations designed to prevent the sexual transmission of pathogens, particularly HIV [12]. The choice of the dosage form and its composition can have a major influence on the efficacy of the active ingredient. Although vaginal microbicides were initially formulated mainly as gels, interest has grown in

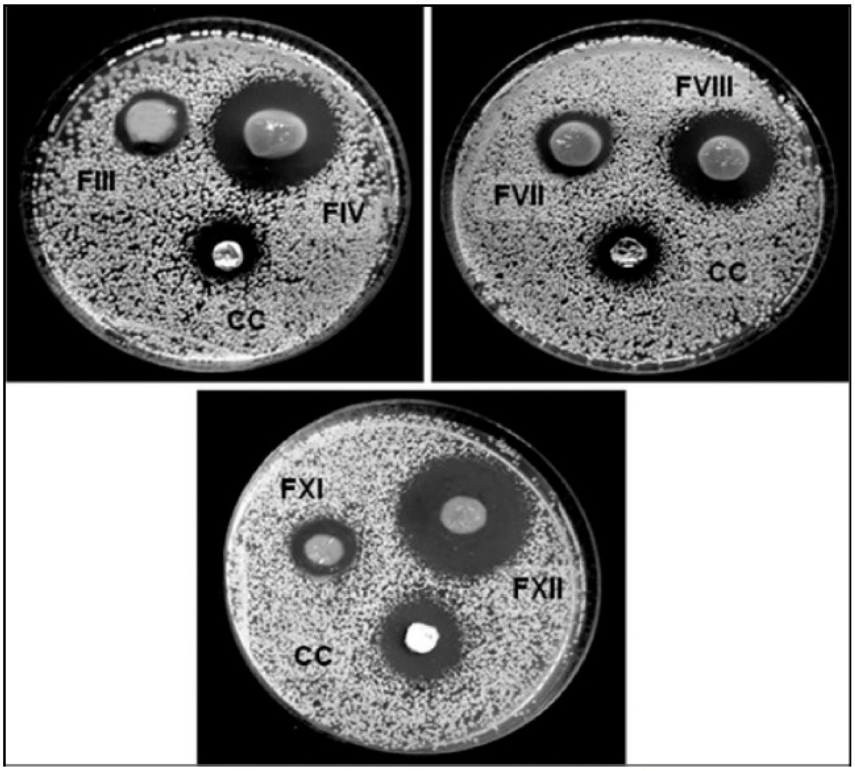

Fig. 20. Halos of growth inhibition produced by films without ECN (FIII, FVII, FXI) and loaded with ECN (FIV, FVIII, FXII) and a commercial cream (CC) in a culture of *Candida krusei*. Reprinted by permission of Springer Nature from [122], Copyright (2012).

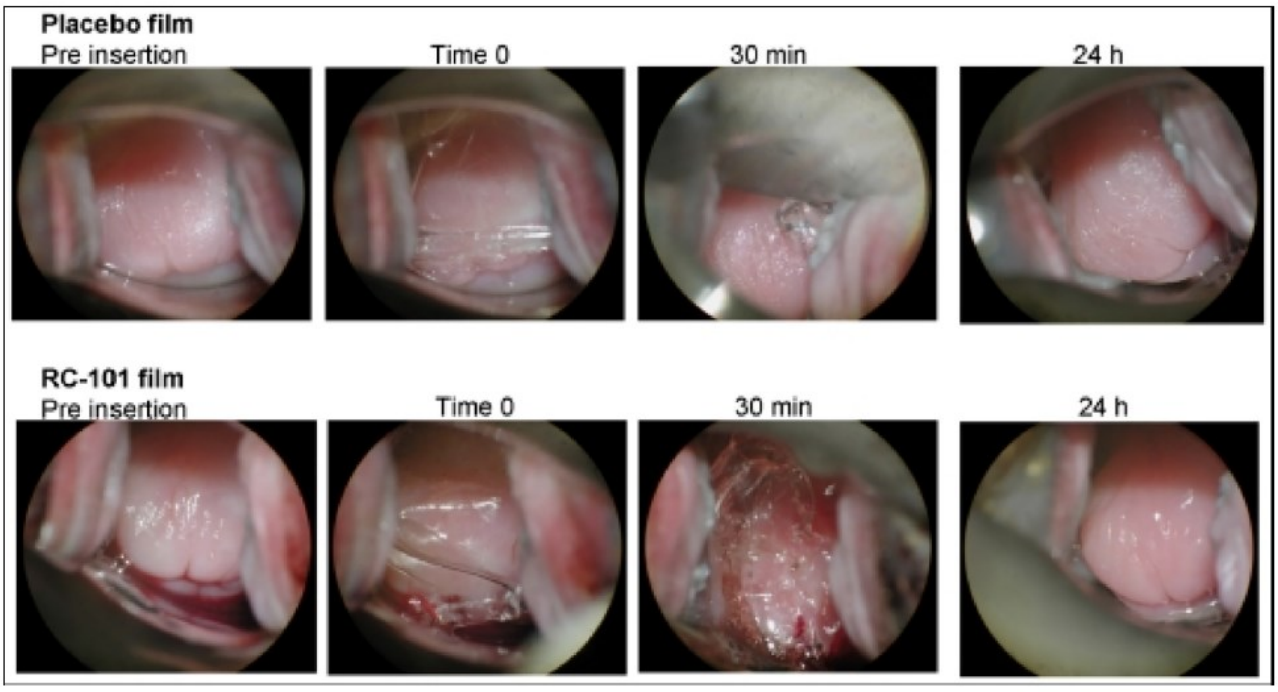

Fig. 21. Colposcopy of the cervicovagina of non-human primates revealing no adverse effects of quick-dissolving films containing no drug (placebo) or the anti-retroviral peptide RC-101 (2 mg per film). Films (27.5 mm × 33.5 mm) comprised PVA, PVA and glycerol (6:0.12:3). Images from colposcopic examination of cervicovaginal mucosa of a representative pigtailed macaque obtained prior to film insertion, at the time of insertion (Time 0), and 30 min and 24 h after film insertion. Note the absence of mucosal aberrations at all time points with either RC-101 films or placebo films. Reprinted from [125] under the terms of the Creative Commons Attribution License 4.0 (Copyright 2020 Cole et al.; doi: https://doi.org/10.1371/journal.pone.0015111).

alternative pharmaceutical forms such as vaginal films [1].

The first references to vaginal films for HIV prevention correspond to the inclusion of surfactants such as sodium dodecyl sulphate (SDS) in order to disrupt the viral membrane. We found a film formulated with a mixture of Carbopol and HPMC using polyethylene glycol as a plasticiser, which contains 20–30 mg of SDS [8]. Vaginal films have also been developed with cellulose acetate phthalate – which has been proven to be capable of adsorbing the virus on its surface – and HPC, plasticized with glycerol. Their *in vitro* activity against HIV has been evaluated [126].

A current trend in anti-HIV microbicides involves the inclusion of antiretroviral drugs. For example, films are commonly prepared with tenofovir (TFV), a nucleotide reverse transcriptase inhibitor. Fast-dissolving films have been developed with cellulose derivatives plasticised with glycerol. The amount of TFV contained in these films is 10 or 40 mg; they have been shown to be safe in clinical trials and have a similar drug release profile to gels [77,78]. The sustained release of TFV has also been achieved with blend vaginal films combining HPMC and zein. When plasticised with polyethylene glycol, these films released the drug in a sustained manner in *in vitro* trials for 120 h [24]. Another option is the preparation of bilayer films: one layer of natural gum (tragacanth or xanthan gum) supplies mucoadhesive properties, while the other layer, formed by EC, ensures the *in vitro* controlled release of TFV over 2 weeks [41]. Intermediate behaviour has been obtained with mixed HPMC and PVA films containing nanoparticles loaded with this drug. A fast release of 30% TFV was achieved in 15 min, followed by 24 h of sustained release [38]. The pH-dependent release of this antiretroviral has also been explored. Bilayer chitosan and Eudragit® S100 film plasticised with organic acids was able to retain the drug until the medium was alkalinised [43].

Another antiretroviral frequently included in films is dapivirine (DPV), a non-nucleoside reverse transcriptase inhibitor. The group of Rohan et al. has developed films containing 1.25 mg of this drug. The films were fabricated by solvent casting, based on a blend of PVA and HPMC plasticised with polyethylene glycol [81], and were evaluated in clinical trials, where they demonstrated their safety. They are well tolerated by women, and no severe adverse effects were observed after administration [127]. It was also found that the same concentrations in plasma, cervicovaginal fluid and cervical tissue were achieved after administration as in gels with the same dose [128]. This group also explored the possibility of fabricating DPV-loaded films by hot melt extrusion as an alternative. These films have very similar characteristics to the ones prepared by solvent casting [108]. The combination of DPV with contraceptive drugs in the same films has also shown good results when evaluated in animals, namely regarding retention and distribution in the vagina (Fig. 22) [124].

Films loaded with numerous other antiretrovirals have also been formulated for this purpose. Ghosal et al. developed films containing abacavir. Films from combinations of HPMC with sodium alginate or PVP were developed by the solvent casting method, showing controlled release of the drug in *in vitro* trials, and safety and suitable drug absorption when administered to animals [72,129]. UAMC01398, a non-nucleoside reverse transcriptase inhibitor, has been incorporated into HPMC films plasticised with polyethylene glycol 400, achieving fast-dissolving films that dissolve in less than 10 min [9]. Another drug from this family is IQP-0528. PVA-based films released this antiretroviral in 30 min, and were stable and non-toxic in *in vitro* trials [82]. The films proved to be safe in macaques, and achieved high levels of the drug in the mucosa [130].

Quick-dissolving films were also chosen to formulate RC-101, an antiretroviral peptide. HPMC and PVA were again chosen as film-forming ingredients and were plasticised with glycerol [80]. Strong activity and low cytotoxicity were demonstrated in an organ culture [131]. The films were also safe in macaques and the drug was still active 5 days after administration [125]. Small interfering ribonucleic acid (siRNA)-nanoparticles have also been successfully incorporated into vaginal films. These films are non-toxic and have suitable mechanical properties [69].

Films have also been produced with a combination of drugs, and both monolayer and multilayer films have been designed to release several antiretroviral drugs. Some examples include the combination of TFV with emtricitabine (incorporated into PVA and pectin films [18]), with efavirenz (co-delivered from mixed HPMC and PVA film [19]) or with DPV (on PVA-based films [121]). The combination of maraviroc, TFV or DPV was also widely studied [99], as well as the combination of 4′-Ethynyl-2-fluoro-2′-deoxyadenosine with 5-chloro-3-phenylsulfonylindole-2-carboxamide [74]. These films proved to have antiviral activity when evaluated in ectocervical explants.

Fig. 22. Tissue retention and distribution of bioadhesive film and quick-dissolving film in a macaque model. Representative colposcope image for blue dye loaded bioadhesive film and quick-dissolving film in pigtailed macaque vaginal compartment (red star marks the last presence of film in vaginal cavity). Reprinted from [124] under the terms of the Creative Commons Attribution License 4.0 (Copyright 2020 Li et al.; doi: https://doi.org/10.3390/pharmaceutics12010001). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

5.2. Treatment of vulvovaginal candidiasis

Vulvovaginal candidiasis is a mucocutaneous yeast infection caused by several *Candida* spp. that affects 70–75% of women at least once during their lifetime, and at least half of them will suffer recurrences [132]. Although current treatments — mostly based on azole compounds — can be administered [133], films are attracting interest as an alternative in this field [134]. Recent studies have proposed some formulations to deliver antifungal drugs [17,67].

Many of the films developed for this purpose are based on chitosan, as this polymer has also been reported to have antimicrobial activity. For example, films based on chitosan — alone and combined with Carbopol — were prepared by solvent casting to deliver ecoconazole nitrate (ECN). They were shown to have suitable mechanical properties and good antifungal activity against both *C. albicans* and *C. krusei*. The antifungal activities of these films were higher than the activity from a commercially available ECN cream, which may be explained by the synergistic effect between ECN and chitosan [122]. Mucoadhesive vaginal films were also prepared by the solvent-casting method to incorporate fluconazole (FLZ). Chitosan and pectin were used as film-forming polymers and glycerol as a plasticiser in these films, which were loaded with 150 mg of FLZ. The addition of dimethyl sulfoxide was tested as a permeation enhancer. The optimized batch showed better controlled drug release than a FLZ gel on the market (Flucos®) and similar *in vitro* inhibitory activity against *C. albicans*. Both systems presented a similar behaviour in regard to *Lactobacillus* growth. *In vivo* irradiation studies performed in rabbits showed no signs of irritation or inflammation after 3 days. This film therefore appears to be a safe alternative to current dosage forms [123]. Calvo et al. also developed films based on chitosan — alone and combined with HPMC —, plasticised with polyethylene glycol 400 and containing tioconazole (TCZ). TCZ-loaded films showed greater inhibition of *C. albicans* than free TCZ or TCZ ovules, while presenting a controlled release of the drug. Chitosan-based films showed some cytotoxicity, whereas blend films were noncytotoxic to cells. The TCZ-loaded film composed of chitosan and HPMC was therefore deemed a promising alternative for the delivery of TCZ [67].

However there are also alternative films that are not based on chitosan. A polymer film comprising PVA and sodium alginate was prepared by the solvent casting method to deliver clotrimazole (CLZ). The film did not show any significant cytotoxicity and was capable of inhibiting the growth of both *C. albicans* and *C. dubliniensis* at 250 μg/mL, killing both strains at 250 and 500 μg/mL respectively [135]. Other examples of films loaded with CLZ are based on HPC and sodium alginate, which achieved rapid disintegration in just 18 min. This study also proved that isopropyl myristate could be used as a permeation enhancer. The *in vitro* permeability test showed that 77% CLZ was able to diffuse in 6 h. The films also demonstrated *in vitro* antifungal activity against *C. albicans* and had no effect on the growth of *Lactobacillus* [71]. Other drugs have also been evaluated for the same purpose. One work assesses itraconazole release from films prepared based on different polymers (HPC, HEC or carrageenan). The films were assessed *in vitro* and proved to have good mechanical properties, as well as being retained in the vaginal mucosa for up to 8 h; they were also noncytotoxic and did not affect the normal vaginal microbiota [27]. Sustained release strategies have also been developed: vaginal films based on HPMC and including FLZ achieved *in vitro* drug release over 12 h [76].

Finally, Bassi and Kaur worked on the development of vaginal films loaded with nystatin, a polyene antifungal drug. Films based on a carboxymethyl derivative of fenugreek gum — a polysaccharide obtained from the seeds of *Trigonella foenun-graecum* [136] — were prepared with glycerol as plasticiser. Nystatin was included at 100,000 units/cm^2. The optimized film presented a fast release profile and delivered all the drug in 5 h. The film proved to be nontoxic *in vitro*, and nontoxic and non-irritant to the vaginal mucosa in *in vivo* studies in female Wistar rats. *In vivo* antifungal studies showed a decline in the *C. albicans* count — introduced intravaginally in the rats — from 10^8 to 3×10^2 colony-forming units (CFU)/mL, over a period of 14 days. The results indicate the antifungal activity of the proposed film against *C. albicans* [17]. A subsequent study was carried out by the same group in order to load this same drug into bioadhesive films based on tamarind seed polysaccharide. *In vitro* studies showed this film was able to release about 74% of the drug and had a suitable residence time of 8.5 h. Tamarind seed polysaccharide has been reported to have antifungal activity [137], so one of the aims of this study was to investigate whether it had a synergistic antifungal effect with nystatin. Both loaded and unloaded films were subject to antifungal activity tests against *C. albicans*, but only loaded films presented the appropriate antifungal activity, demonstrating that tamarind seed polysaccharide does not produce an antifungal synergistic effect. The proposed system appears to be a fitting candidate for the vaginal delivery of drugs [15], although further studies, namely cytotoxicity evaluation and eventually *in vivo* tests, are required.

5.3. Treatment of bacterial vaginosis

Another application of drug-loaded films for vaginal administration is the treatment of bacterial vaginosis. Research in this field is scarce. However, one published work describes bioadhesive films of HPMC and xanthan gum, including clindamycin, to treat bacterial vaginosis [73]. The optimal batch showed good mechanical properties and retention on vaginal mucosa for 8 h, and the formulation was non-toxic in *in vitro* trials.

5.4. Treatment of female sexual dysfunction

Nitric oxide has been proposed to increase vaginal blood perfusion and thus increase sexual arousal. However, the low half-life of the drug leads to a short action time. Films have therefore been developed containing S-nitrosoglutathione (GSNO), a nitric oxide donor that decomposes with temperature and releases nitric oxide [10]. Carbomer and HPMC were selected as ingredients to form the film, which was plasticised with polyethylene glycol. It was demonstrated that the higher the drying temperature, the lower the loading efficiency, as the drug decomposed with the rise in temperature; this problem was overcome by drying films at 15 °C under reduced pressure. The films were shown to release nitric oxide *in vitro* in a sustained manner for 12 min, and to increase the intracellular expression of cyclic guanosine monophosphate (cGMP). *In vivo* studies showed that films successfully increased vaginal blood flow in rats (Fig. 23).

5.5. Contraceptives

The development of films for contraceptive purposes has a long history and can be traced to the 1960s. The first attempts were reported by Hotay in 1968, namely a formulation called C-Film comprising a PVA-based film incorporating the spermicide nonylphenoxypolyethoxyethanol [138]. However, the clinical trial results were poor and its development was discontinued [139]. A film containing the spermicide nonoxynol-9 is currently available on the market and is used as a contraceptive [1]. Other films have subsequently also been developed with nonoxynol-9, with PVA or HPMC as polymers (aimed at rapid dissolution in the vaginal environment), containing between 100 and 130 mg of this spermicide [22].

Research into the development of contraceptive films is still ongoing. Films containing benzalkonium chloride — a substance that has been shown to immobilize sperm — have also been evaluated, and have been shown to have an efficacy comparable to nonoxynol-9 film already on the market [140]. Another option is the preparation of films loaded with 300 mg of sodium polystyrene sulfonate, an agent with both contraceptive and antimicrobial properties, formulated for rapid dissolution in the vaginal environment and which could not only prevent

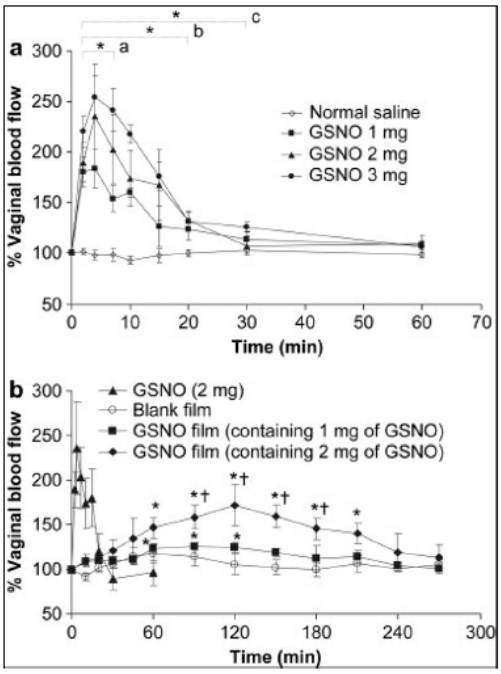

Fig. 23. The change of vaginal blood flow with the treatment of S-nitrosoglutathione (GSNO) and the GSNO films. (a) Normal saline ($n = 5$), GSNO 1 mg ($n = 3$), GSNO 2 mg ($n = 4$), GSNO 3 mg ($n = 4$), (*) denotes $p < 0.05$ *versus* baseline ([a]GSNO 1 mg, [b]GSNO 2 mg, [c]GSNO 3 mg). (b) GSNO 2 mg ($n = 4$), blank film ($n = 4$), GSNO film containing 1 mg of GSNO ($n = 3$), GSNO film containing 2 mg of GSNO ($n = 3$). All data are expressed as mean ± standard error of the mean. (*) denotes $p < 0.05$ *versus* baseline; † denotes $p < 0.05$ *versus* blank film. Reprinted from [10], with permission from Elsevier, Copyright (2009).

pregnancy, but also sexually transmitted diseases [141]. However, the clinical demonstration is not available.

In regard to hormonal contraceptive methods, a film loaded with levonorgestrel alone and in combination with DPV has recently been developed. Tests in animals showed that the film was able to release the drug in a sustained manner for several days [124].

Finally, a recent reference in the literature is a contraceptive film based on PVA and an occlusive polymer named RISUG® (reversible inhibition of sperm under guidance), which offers a non-hormonal and completely biocompatible method to prevent conception, since it has been proven not to cause significant toxicity in *in vivo* studies [142]. However, its contraceptive efficiency has not been clinically demonstrated.

6. Concluding remarks

Vaginal films are a pharmaceutical form that is attracting increasing interest from researchers. Their versatility in terms of usable raw materials and fabrication techniques, and their potential advantages over other vaginal administration dosage forms, points to their potential for the vaginal administration of drugs. The multiple advantages that can be claimed for vaginal films guarantee their further development. However, their actual use by pharmaceutical manufacturers is low, as evidenced by the fact that there are only three vaginal films on the market. The concept of vaginal film has evolved in recent years; while initially these systems were only conceived as dispersible films for rapid drug administration, they are now also being considered for controlled drug release. The possibility of using vaginal films as microbicides for the prevention of HIV transmission is undoubtedly the most widely studied of the applications included in this review due to the absence of effective alternatives today. One particular lesson from the field is that appearances matter. Adherence is highly dependent on whether women like films or not, and are willing to use them consistently. This has indeed been the Achilles' heel in the field of microbicides. Despite technological challenges and scale-up issues, we foresee that the future will undoubtedly lead to the fabrication of smart vaginal films, which will allow the treatment or prevention of diseases to be adapted to therapeutic needs, thus facilitating the day-to-day life of patients. Multilayer films, where each layer provides different properties to the dosage form, hold considerable promise for the development of "smart" films and will surely see substantial advances in the years to come.

Declaration of competing interest

None.

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Author contributions

All the authors have approved the final article.

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CAPÍTULO VII

DEVELOPMENT OF MUCOADHESIVE VAGINAL FILMS BASED ON HPMC AND ZEIN AS NOVEL FORMULATIONS TO PREVENT SEXUAL TRANSMISSION OF HIV

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Development of mucoadhesive vaginal films based on HPMC and zein as novel formulations to prevent sexual transmission of HIV

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ABSTRACT

Although vaginal films were initially developed for a fast release of the drug, with the adequate formulation they can also be useful for sustained release. The latest strategies for the prevention of the sexual transmission of HIV have moved towards sustained-release dosage forms, so films may be an effective strategy that could also improve the patient's comfort. A hydrophilic polymer (hydroxypropylmethyl cellulose) and an amphiphilic polymer (zein) have been evaluated for the development of Tenofovir sustained-release vaginal films. The modification of the film's properties by the inclusion of polar (glycerol and polyethylene glycol 400 (PEG)) and amphiphilic (tributyl citrate and oleic acid) plasticisers was also evaluated. The films' physicochemical and mechanical properties were determined. The *in vitro* release of Tenofovir from the films and their bioadhesive capacity and behaviour in simulated vaginal fluid were also assessed. The combination of hydroxypropylmethyl cellulose and zein in films (ratio 1:5), with the inclusion of PEG (40% w/w) proved not only to have excellent mechanical properties, but was also able to release TFV in a sustained manner for 120 h and remain attached to biological tissues throughout this time. This film could be an interesting option for the prevention of sexual transmission of HIV.

1. Introduction

Vaginal films are an unusual dosage form that have gained in importance in recent years, as endorsed by the numerous research works in the literature that assess the inclusion of different drugs in these films, mainly hormones, contraceptives, antifungals and antivirals (Mishra et al., 2016; Garg et al., 2005; Akil et al., 2011). This last option, the inclusion of antivirals in vaginal films, is of special interest for preventing the sexual transmission of the human immunodeficiency virus (HIV), given the urgent need to develop an effective vaginal microbicide (Doggett et al., 2015; Notario-Pérez et al., 2017). Women, especially in the sub-Saharan area, are by far the population group that is most affected by HIV (there are currently 18.8 million women and girls living with HIV and 870,000 are infected each year) (UNAIDS, 2017), so that a vaginal film that women could use by themselves (without the need for a man's cooperation) and that protects them from acquiring the virus, would be a very important step for eradicating the HIV epidemic, one of the priority objectives of the United Nations.

According to the definition of Machado et al., films are solid dosage forms that are capable of quickly dissolving in the vaginal fluid, avoiding leakage and messiness (Machado et al., 2013). The vast majority of films for preventing the transmission of HIV in the literature clearly respond to this definition. Among the antiretrovirals used in the manufacture of these films are nucleoside reverse transcriptase inhibitors (NRTIs) such as Abacavir (Ghosal et al., 2016; Ghosal et al., 2014) or 4′-ethynyl-2-fluoro-2′-deoxyadenosine (EFdA) (Zhang et al., 2014), and non-nucleoside reverse transcriptase inhibitors (NNRTIs) such as IQP-0528 (Srinivasan et al., 2016; Ham et al., 2012) or UAMC01398 (Grammen et al., 2014). Formulations can also be manufactured with a combination of drugs with different mechanisms of action, for example a film that combines Tenofovir (TFV) and Dapivirine (DPV) (Akil et al., 2014), another film with EFdA and 5-chloro-3-phenylsulfonylindole-2-carboxamide (CSIC) (Zhang et al., 2015); or a recent study that compares the combination of DPV and TFV but also

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combines them with a virus entry inhibitor (Maraviroc) (Akil et al., 2015). All these films have been designed for rapid dissolution in the vaginal environment, thereby achieving the release of the drug in a short period of time (from under ten minutes to six hours).

The most common polymers for obtaining these films are hydroxypropylmethyl cellulose (HPMC) (Mishra et al., 2016; Akil et al., 2011; Grammen et al., 2014; Ghosal et al., 2016; Ghosal et al., 2014; Zhang et al., 2014; Zhang et al., 2015; Akil et al., 2015; Kumar et al., 2013; Machado et al., 2016) and polyvinyl alcohol (PVA) (Akil et al., 2011; Zhang et al., 2014; Ham et al., 2012; Machado et al., 2016; Gu et al., 2015; Akil et al., 2014; Zhang et al., 2015; Akil et al., 2015), although films have also been developed with other polymers such as polyvinyl pyrrolidone (PVP) (Ghosal et al., 2016; Akil et al., 2015), pullulan (Zhang et al., 2015), sodium alginate (Mishra et al., 2016; Ghosal et al., 2014), carrageenan (Gu et al., 2015), sodium carboxymethyl cellulose (Akil et al., 2015) and hydroxyethyl cellulose (Akil et al., 2015). All these polymers are characterised by being water-soluble and capable of dissolving or gelling in the presence of vaginal fluid. The most common feature is that each film is not formed by a single polymer but by combinations of different polymers. We highlight HPMC, a semi-synthetic polymer derived from cellulose, which is a non-ionic water-soluble polymer able to improve the biopharmaceutical properties of many drugs (Riekes et al., 2014). Its swelling and dissolution properties in aqueous medium make it an excellent vehicle to control the drug release (Riekes et al., 2014; Notario-Pérez et al., 2017), which is why it is also common to find not only films manufactured with HPMC, but also gels and tablets (Tuğcu-Demiröz et al., 2015; Hiorth et al., 2014).

The use of polymers in the development of pharmaceutical formulations (and especially in vaginal films) requires the study of plasticising substances, which endow the formulation with the appropriate physicochemical characteristics. Nevertheless, despite the number and diversity of these substances, only a few plasticisers have been approved for pharmaceutical applications. Natural plasticisers such as vegetable oils are particularly recommended, as they are characterised by having low toxicity and migration parameters, which is very important for their incorporation into films. The references to films in the literature mainly use glycerine (Akil et al., 2011; Ghosal et al., 2014; Zhang et al., 2015), polyethylene glycol (PEG) (Mishra et al., 2016; Akil et al., 2011; Grammen et al., 2014; Zhang et al., 2015; Akil et al., 2015; Kumar et al., 2013) or propylene glycol (Mishra et al., 2016; Akil et al., 2011; Zhang et al., 2014) as plasticising substances, which are incorporated into the formulation in variable proportions (we found from 5% to 40%).

However, although most films are designed for rapid dissolution in the presence of vaginal fluid, we also found a film capable of releasing TFV for 24 h thanks to the development of TFV-loaded nanoparticles (Machado et al., 2016), and a film able to release DPV over seven days (Bunge et al., 2016), due to the low solubility of this drug in vaginal fluid. The development of these formulations is in line with the new trend in research into vaginal microbicides, which aims to stagger the administration of the dose to achieve a greater adherence to use and consequently an increased protective efficacy (Notario-Pérez et al., 2017; Hankins and Dybul, 2013). This search for sustained-release formulations has made it possible to manufacture films based on non-water-soluble polymers which could partially isolate the drug from the vaginal fluid, thus achieving a slower release. We highlight zein, an amphiphilic corn-derived protein that is insoluble in aqueous medium, soluble in alcoholic solutions and stable in a wide range of pH (which makes it very valuable for the manufacture of vaginal films, as it would remain unchanged in the presence of vaginal fluid –pH approximately 4.2– and seminal fluid –pH approximately 7.9) (Zhang et al., 2016; Karthikeyan et al., 2012). Although its most frequent use for pharmaceutical purposes is to obtain solid-form coatings, matrix tablets have also been developed in recent years (Bouman et al., 2016) and even zein-based nanoparticles (Podaralla and Perumal, 2012). Zein's excellent film-forming properties and its low permeability to water and gas (Corradini et al., 2014) give this polymer great potential for the development of therapeutic films. However, pure zein films have the limitation of their poor mechanical properties, since they are extremely fragile and inflexible (Zhang et al., 2015), so it is almost essential to combine it with plasticising agents. Since it is an amphiphilic polymer, the inclusion of amphiphilic substances such as fatty acids (oleic acid, palmitic acid, lauric acid, etc.) or citrate derivatives (tributyl citrate (TBC), triethyl citrate, etc.) has also been evaluated, in addition to all the polar plasticisers mentioned above (glycerol, polyethylene glycol, etc.) (Zhang et al., 2015; Masamba et al., 2016; Serna and Filho, 2015).

The main advantage that vaginal films offer, compared to other formulations that can be developed for this purpose –such as gels or tablets–, are the absence of leakage during use, discreet and comfortable use for women, high bioadhesion to vaginal mucosa due to their high surface and little weight, minimal packaging and reduced waste (Notario-Pérez et al., 2017).

Based on the above, the aim of this work is to develop a vaginal film that allows the sustained release of TFV. The possibility is assessed of obtaining films from a hydrophilic polymer (HPMC), an amphiphilic polymer (zein), and the combination of both, studied here for the first time. Various polar (glycerol and PEG 400) and amphiphilic (oleic acid and TBC) plasticising agents are also tested.

2. Materials and methods

2.1. Materials

Tenofovir (TFV, lot: FT104801401) was supplied by Carbosynth Limited (Berkshire, UK). Hydroxypropylmethyl cellulose—Methocel® K 100 M (HPMC; lot: SB13012N31) was kindly supplied by Colorcon Ltd. (Kent, UK). Zein (lot: SLBL9380V), Kollisolv® PEG E 400 (PEG, lot: BCBQ6662V), tributyl citrate (TBC, lot: BCBP4709V) and oleic acid (lot: BCBR1202V) were acquired from Sigma-Aldrich (St. Louis, MO, USA). Glycerol (lot: 0000539368) was acquired from Panreac (Barcelona, Spain).

Methanol, ethanol and all other reagents used in this study were of analytical grade and used without further purification. Demineralised water was used in all cases.

2.2. Film preparation

The films were prepared using HPMC, zein or a mixture of both; and glycerol, PEG 400, TBC or oleic acid was incorporated as plasticising agent. The films were manufactured by the solving cast method in a mixture of water/methanol, using 43 mm-diameter non-adhesive silicone templates. The solution/dispersion of the drug, polymer and plasticiser was placed on these templates, and the solvent was evaporated at room temperature, obtaining thin polymeric films. Raw materials of every unit were individual weighed to ensure that there is no variation among drug loaded in each film.

2.3. Film characterisation

The prepared films were visually assessed to verify their appearance, flexibility and fragility. Some proved difficult to unmould due to their extreme fragility, while others were too greasy. These films and others that were not smooth or showed surface cracks were discarded. The films selected for further trials are shown in Table 1.

2.3.1. Pliability

The pliability of the films was studied in greater detail. All the formulations were manually tested, and pliability was rated as:

- Completely flexible: can be folded in any way without breaking, even back on itself, and recover its original shape.
- Flexible: can be folded forcefully without breaking, although if

Table 1
Composition (mg) of the films selected for further trials.

Batch	HPMC	Zein	Glycerol	PEG 400	TBC	Oleic acid	TFV
H100	100						30
H100G40	100		40				30
H100G80	100		80				30
H100P40	100			40			30
H100P80	100			80			30
H200	200						30
H200G40	200		80				30
H200G80	200		160				30
H200P40	200			80			30
H200P80	200			160			30
Z200		200					30
Z200T40		200			80		30
Z200T80		200			160		30
Z200O40		200				80	30
Z200O80		200				160	30
Z500		500					30
Z500T40		500			200		30
Z500T80		500			400		30
Z500O40		500				200	30
Z500O80		500				400	30
H100/Z200	100	200					30
H100/Z200G40	100	200	120				30
H100/Z200G80	100	200	240				30
H100/Z200P40	100	200		120			30
H100/Z200P80	100	200		240			30
H100/Z500	100	500					30
H100/Z500G40	100	500	240				30
H100/Z500G80	100	500	480				30
H100/Z500P40	100	500		240			30
H100/Z500P80	100	500		480			30

folded back on itself, it has problems recovering its initial shape.
- Slightly flexible: can bend to a certain point before breaking.
- Rigid: cannot be folded, but given its thickness, it does not break unless great force is applied.
- Fragile: breaks at any attempt to fold.
- Very fragile: breaks easily, even during unmoulding.

2.3.2. Resistance and elasticity

The resistance and elasticity of the films was measured using a TA.TXTplus Texture Analyser (Stable Micro Systems, Surrey, UK). An experiment was designed to analyse the force applied and the deformation of the film. The film was fixed on a plate with a hole in the middle. A probe was lowered onto the film, and a quantified force was applied, causing the film to deform through the hole until breaking. This test was performed in quadruplicate.

2.3.3. Thermal analysis

Thermogravimetric analysis (TGA) was performed in a SDT-Q 600 TA instruments TG/DTA analyser. A small piece of film (about 5–10 mg of sample) was placed in a pinholed aluminium sample pan with a lid and heated in atmospheric air to between 20 °C and 500 °C. TGA of raw materials was also evaluated for a better understanding of the results.

2.3.4. Bioadhesion test

The films' adhesiveness to biological samples was evaluated through an adaption of a previously described method (Notario-Pérez et al., 2017). Bioadhesion to biological tissue was assessed using animal skin, which was fixed with acrylic adhesive to a stainless-steel plate. The film was placed on top of the skin –previously wetted with SVF– and pressure of 500 g was applied for 30 s. The plate was then placed in a beaker with SVF at an angle of 60°, in a thermostatised shaking water bath (Selecta® UNITRONIC320 OR, Barcelona, Spain) at 37 °C and 15 opm. All batches of film were tested in duplicate. The samples were visually observed at regular intervals, and the average time taken by the samples in each batch to become detached was established as the bioadhesion time.

2.3.5. Film behaviour in simulated vaginal fluid

The behaviour of the films in the presence of vaginal fluid was simulated in a study in which the films were placed on stainless steel discs and submerged in simulated vaginal fluid (SVF) (Owen and Katz, 1999) inside a beaker. The beakers were then placed inside a thermostatised shaking water bath (Selecta® UNITRONIC320 OR, Barcelona, Spain) at 37 °C and 15 opm to simulate physiological characteristics. Each film was tested in triplicate.

At given times, the discs –with the films on them– were removed from the medium, placed on filter paper to eliminate excess liquid, and weighed. The water capture (WC) –expressed as the percentage of the weight of water of the total film weight– was calculated according to Eq. (1):

$$WC = \left(\frac{F_w - F_d}{F_d} \right) \quad (1)$$

where *Fw* and *Fd* correspond to the wet and dry film weights respectively.

Since neither the plasticiser nor the drug are able to capture water, this measure was standardised and the adjusted water capture (AWC) was determined, where the WC is related to the amount of polymer (AP) included in the film, as expressed in Eq. (2):

$$AWC = \frac{WC \cdot Fd}{AP} \quad (2)$$

where *WC*, *Fd* and *AP* correspond respectively to the water capture determined by Eq. (1), the dry film weight, and the amount of polymer (HPMC and/or zein) included in the film.

2.3.6. Release study

The film's behaviour following TFV release was evaluated by placing it in the bottom of a borosilicate glass bottle with 80 mL of SVF, which was then placed in a thermostatised shaking water bath (Selecta® UNITRONIC320 OR, Barcelona, Spain) at 37 °C and 15 opm. The release studies were performed in sink conditions since according to previous studies the solubility of TFV in SFV at room temperature is 4 mg/mL (Notario-Pérez et al., 2018).

Samples were taken and filtered at given times, and the medium was replaced to ensure constant SVF volume. The amount of TFV released was quantified by UV spectroscopy in a Shimadzu® UV-1700 spectrophotometer (Kyoto, Japan) at a wavelength of 260 nm. This test was also performed in triplicate for each batch of films.

The experimental data obtained were fitted to drug release model-dependent methods that can explain the release behaviour of TFV from the films (zero order, Higuchi, Hopfenberg and Ritger-Peppas) (Costa and Sousa Lobo, 2001; Mamani et al., 2012).

The zero order model could apparently be applied to pure zein films, since this model represents a dosage form where the drug is slowly released while the formulation remains unchanged. It is expressed by Eq. (3), where Q_0 is the drug included in the film, Q_t the amount of drug released at time t and K_0 the zero order release constant.

$$Q_0 - Q_t = K_0 \cdot t \quad (3)$$

Pure HPMC films will predictably release the drug as a matrix formulation. These batches are considered to have the best fit to the Higuchi model (Eq. (4)), which is useful when the drug is released by a diffusion mechanism based on Fick's law; and the Hopfenberg model (Eq. (5)), applied to surface-eroding dosage forms.

$$Q_t = K_H \cdot t^{1/2} \quad (4)$$

The Higuchi equation is shown in Eq. (4), where Q_t is the amount of drug released at time t and K_H is the Higuchi dissolution constant.

$$\frac{Q_t}{Q_0} = 1 - [1 - k_{HF} \cdot t]^{n_H} \quad (5)$$

The Hopfenberg model is expressed as Eq. (5), where Q_t is again the drug released at time t and Q_0 the total drug included. K_{HF} is the Hopfenberg rate constant, which includes the expression $K_0/C_0 a_0$, where K_0 is the erosion rate constant, Q_0 is the initial drug concentration in the dosage form, and a_0 is half the initial system thickness. n_H is the Hopfenberg exponent, which is related to geometry and has a value of 1 (for slab), 2 (for cylindrical) or 3 (for spherical) depending on the system.

For our compacts, where n_H is equal to 1, this expression can be summarised in Eq. (6):

$$\frac{Q_t}{Q_0} = - k_{HF} \cdot t \quad (6)$$

Finally, since it is more difficult to predict the drug release behaviour from films combining HPMC and zein, the experimental results were also adjusted to the Ritger-Peppas model (Eq. (7)). This approximation of the classic Korsmeyer-Peppas model allows the drug release mechanism to be defined from a thin film according to the value of n, and is a Fickian diffusion model when $n \leq 0.5$, an anomalous (non-Fickian) transport when $0.5 < n < 1$ (there are both diffusion and erosion mechanisms), a zero order release when $n = 1$, and a case II transport if $n > 1$ (which implies a structural modification of the polymer matrix) (Chime et al., 2013).

$$Q_t/Q_\infty = K_{RP} \cdot t^n \quad (7)$$

In the Ritger-Peppas equation, Q_t/Q_∞, K_{RP} and n are the fraction of drug released at time t, a constant which includes the geometric and structural characteristics of the system and the release exponent respectively. The portion of the curve used for the fit to this model is where $Q_t/Q_\infty < 0.6$.

The experimental data were also adjusted to a model-independent index, the similarity factor (f_2), which is calculated through Eq. (8) (Moore and Flanner, 1996).

$$f_2 = 50 \times \log\left\{\left[1 + \left(\frac{1}{n}\right)\sum_{j=1}^{n} W_j |R_j - T_j|\right]^{-0.5} \times 100\right\} \quad (8)$$

This model indicates the similarity between a reference and a test profile; when $f_2 > 50$ the profiles can be said to be similar, and when $f_2 < 50$ the profiles are dissimilar (Notario-Pérez et al., 2018). In this equation, n is the number of samples for each dissolution test, R_j and T_j are the drug release percentage at each time for the reference and test product respectively and W_j is a weight factor.

2.3.7. Cytotoxicity assessment

Three human cell lines were used to evaluate cytotoxicity: a lymphoblastic cell line, MT-2 (Harada et al., 1985), a macrophage-monocyte-derived cell line, THP-1 (ATCC® TIB-202) and a uterus/endometrium epithelial cell line, HEC-1-A (ATCC® HTB-112™) (kindly provided by Maria Angeles Muñoz). All the cells were grown and propagated in RPMI 1640 medium supplemented with 10% (v/v) foetal bovine serum, 2 mM L-glutamine and 50 mg/mL streptomycin at 37 °C with a humidified atmosphere of 5% CO_2. The HEC-1-A cells were detached by removing the medium and rinsing the flask for 10 min with 1 to 2 mL of trypsin 0.25% - EDTA 0.03% solution. The medium was replaced every three days after cell centrifugation at 1500 rpm for 5 min.

Toxicity evaluation. Cell toxicity was measured by CellTiter Glo (Promega, Madison, USA). The materials were suspended in water following a standard method (Krug, 2011). Briefly, the materials were first incubated at 37 °C, 5% CO_2 in PBS 1x for 48 h to obtain the suspensions used to treat the cells. Cells were incubated in 96-well plates at a density of $10{\cdot}10^5$ cells per well (MT-2 and THP-1) and $2{\cdot}10^4$ (HEC-1-A) in complete medium, and then exposed to fresh medium containing different concentrations of zein, glycerol, PEG, TBC or oleic acid suspensions or the same concentration of suspension vehicle as control (PBS 1x in the same conditions). Experiments were done in triplicate and the culture was maintained at 37 °C and 5% CO_2 for 48 h. After incubating for 48 h, the culture media was removed, 50 µL of CellTiter Glo reagent was added to each well, and the supernatants were transferred to a white microplate. Relative luminescence units (RLUs) were measured in a luminometer (Sirius, Berthold Detection Systems). Cytotoxic concentrations 50 (CC_{50}) values were calculated using GraphPad Prism software (non-linear regression, log inhibitor versus response). The results of the cytotoxic assay are shown as the average of at least three individual experiments.

3. Results and discussion

3.1. Film characterisation

HPMC films were prepared containing glycerine and PEG since the plasticisers were perfectly compatible with the polymer. However, TBC and oleic acid showed incompatibility with the HPMC, so were discarded. Zein films perfectly incorporated amphiphilic plasticisers (oleic acid and TBC) but were incompatible with glycerine and PEG. Finally, oleic acid presented incompatibility in the HPMC/zein films, and TBC, although incorporated, did not improve the formulations. The best plasticisers of those tested for mixed films were therefore glycerol and PEG.

TFV physicochemical properties play also a crucial role in film structure, and specially on mixed HPMC/zein films. It is characterized by having a mainly polar surface area. Its water solubility is 1.87 g/L and it is also soluble in methanol. On its structure there are present both hydrogen donors and acceptors, which allow it to establish hydrogen bonds (PubChem, 2019). Since our films are prepared using a mixture of methanol/water as solvent, TFV solubility is ensured. Thus, when raw materials are on dissolution, it is expected that TFV would stablish hydrogen bonds with HPMC. In addition, since the solvent cast method was used to prepare the films, when solvent is evaporated less soluble components are the ones that first precipitate and form the lower layers of the films. HMPC, whose solubility is lower than zein and TFV, will be placed at the bottom of the films, foreseeably in a network with TFV joined by hydrogen bonds. Over this layer, zein will precipitate later, so the drug would be trapped by both polymers. This probably becomes essential for controlled drug release and adhesion of the formulation, since we can establish the hypothesis that there would be a layer of the films with excellent bioadhesive properties due to the presence of HPMC, which in the clinical application would be attached to the vaginal mucosa, and on the other side of the films we would find a layer of zein, which would be the one that would be in contact with the vaginal fluid and through which TFV would have to diffuse, therefore controlling the release of the drug.

3.2. Pliability

The first characteristic evaluated in the different batches of films was their pliability. As can be seen in Table 2, HPMC films are always completely flexible, even in the absence of a plasticiser (Zhang et al., 2018). 100 mg appears to be a better amount of HPMC than 200 mg to obtain a uniform film, since films containing 200 mg tend to have bubbles in their structure. It was also noted that films with 200 mg of polymer and 80% (% w/w) of plasticiser are slightly greasy, while films with the same proportion of plasticiser but only 100 mg of HPMC are not. This is probably due to the structure of the polymer chains, as H100 films are more "two-dimensional" and there is more tension between the chains, so it admits more plasticiser before causing relaxation. However since the surface area is the same, when using 200 mg of HPMC, the polymer chains are distributed in a more "three-

Table 2
Composition of films selected for further trials.

Polymer		Plasticiser		Film characteristics	
Type	Amount (mg)	Type	Amount (mg)	Flexibility	Comments
HPMC	100	–	–	Completely flexible	Slightly folded
		G	40	Completely flexible	
			80	Completely flexible	
		PEG	40	Completely flexible	
			80	Completely flexible	Slightly folded
	200	–	–	Completely flexible	Many bubbles and some holes
		G	80	Completely flexible	With bubbles
					Slightly folded
			160	Completely flexible	With bubblesSlightly greasy
		PEG	80	Completely flexible	Some bubbles
			160	Completely flexible	With bubblesSlightly greasy
Zein	200	–	–	Fragile	
		TBC	80	Very fragile	Some with cracks
			160	Fragile	Slightly greasy
		AO	80	Slightly flexible	
			160	Flexible	Slightly greasy
	500	–	–	Fragile	
		TBC	200	Fragile	
			400	Rigid	Very greasy
		AO	200	Fragile	
			400	Slightly flexible	Very greasy
HPMC/Zein	100/200	–	–	Slightly flexible	
		G	120	Flexible	
			240	Completely flexible	
		PEG	120	Completely flexible	
			240	Completely flexible	
	100/500	–	–	Fragile	
		G	240	Flexible	
			480	Flexible	With bubbles
		PEG	240	Flexible	
			480	Completely flexible	

dimensional" way, so they are more flexible in themselves and do not incorporate so much plasticiser. This excess of plasticiser is what renders these films slightly greasy, as described in Table 2. On the other hand, zein films are very fragile, making it necessary to include a plasticiser (Marcos et al., 2010). Oleic acid proved to be more suitable for zein films, since batches with this plasticiser are more flexible than those with TBC. Large amounts of plasticiser make zein films excessively greasy, and this is especially noticeable in films with 500 mg of zein, confirming that the thicker they are, the less plasticiser is required (when polymer chains can be arranged in a more three-dimensional structure). Despite this improvement, these films are never as flexible as HPMC films, even with the inclusion of larger amounts of oleic acid. HPMC and zein were therefore mixed, and 100 mg of HPMC was included (which proved to be sufficient to give the films flexibility) with both 200 mg or 500 mg of zein. Of these, the batches with PEG as plasticiser were the best in terms of pliability.

3.3. Resistance and elasticity

In order to confirm and quantify the films' pliability, a texture analyser was used to measure the force required to break each batch of films and the distance they can be deformed until rupturing. Both resistance and elasticity must be considered to select the batches with the best mechanical properties.

Among pure zein films, batch Z200 was easily broken (with the application of only 0.57 N, Fig. 1) and the films were only deformed 0.3 mm before rupturing (Fig. 2). Batch Z500 required greater force to break (2.4 N), but was not more elastic. It is therefore confirmed that these films are unsuitable for clinical use due to their poor mechanical properties. The most obvious solution to this problem is the use of plasticising agents such as TBC or oleic acid, which have been demonstrated to be compatible with zein. However, as can be seen in Figs. 1 and 2, the inclusion of TBC barely improves the films' mechanical properties. Neither resistance nor elasticity are improved in batches with 500 mg of zein and TBC, which contradicts previous studies in the literature (Shi et al., 2012). Oleic acid proved to be a good plasticiser for zein films, since it improves their mechanical properties (Budi Santosa and Padua, 1999; Turasan et al., 2018). The best example is batch Z500O80, which can withstand 3.32 N of force and deforms 1.5 mm (improving the elasticity of the Z500 films by 5). Nevertheless, the mechanical properties of this batch are still not acceptable for clinical use.

Batches of HPMC films without plasticiser already have good elasticity (Fig. 2) and resistance properties (Fig. 1), and the more HPMC the film contains, the more force it can resist and the more it can be deformed before rupturing. It was also demonstrated that generally the more plasticiser in the film, the greater its elasticity (Fig. 2). It is therefore clear that the inclusion of glycerol or PEG improves the mechanical properties of HPMC films, confirming the results found in the literature (Zhang et al., 2018). As an example, H100 films can withstand 7.45 N and be deformed 0.9 mm, while this same film with 80% of PEG (batch H100P80) is able to support 32.14 N and be deformed until 5.8 mm.

Films with a HPMC/zein mixture were therefore studied in order to improve the mechanical properties of zein, interspersing the HPMC chains between the zein chains. This mixture, which has previously been studied with positive results as polymer blends in solid dispersions, has never been evaluated in films (Van Ngo et al., 2016). As expected, batch H100/Z200 had better mechanical properties than H100/Z500 due to the higher proportion of HPMC in their structure. These batches are an improvement on the ones with only zein (Z200 and Z500), especially in terms of force resisted (Fig. 1), and even more so with the addition of a hydrophilic plasticiser such as glycerol or PEG. The most encouraging batches contain PEG, and particularly batches

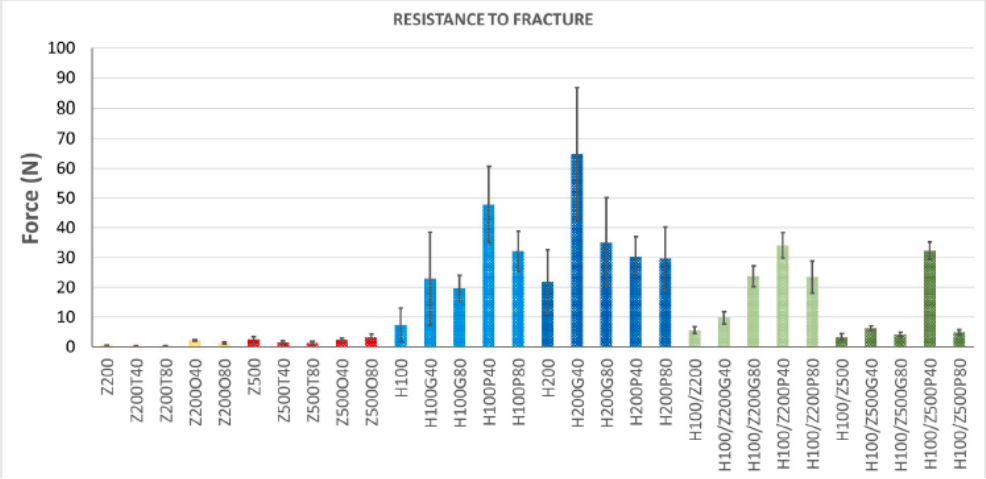

Fig. 1. Results of the fracture resistance of the prepared films, expressed as force (N) required to cause breakage.

H100/Z200P40 and H100/Z500P40, which can withstand over 30 N and are deformed more than 3 mm. Another interesting finding was that these films do not continue to improve with the addition of higher amounts of plasticiser, as despite their greater elasticity they withstand less force before breaking, making them so easily deformable that they cannot maintain a minimal structure.

In general terms, the batches tested can be grouped into two categories according to their resistance to fracture: those that withstand up to 10 N, and those that withstand 20 N or more. Accordingly, we can consider films that support at least 20 N of force to be acceptable for clinical use, so all batches containing only zein are discarded. All batches with HPMC are acceptable except H100, and only four of the mixed batches comply with this requirement: H100/Z200G80, H100/Z200P40, H100/Z200P80 and H100/Z500P40. In terms of elasticity, a minimum of 2 mm of deformation is required to ensure they are not so rigid that they could prove uncomfortable to users. Again, none of the pure zein batches are suitable. It should be noted that all batches containing PEG show excellent elasticity properties, so this is unquestionably the most suitable plasticiser for the films.

3.4. Thermal analysis

A thermogravimetric analysis was performed to detect any possible interactions between polymers and plasticisers that could alter the thermal behaviour of the films. A TGA of the raw materials was done for a better knowledge of the expected outcome, shown in Fig. 3. Although both polymers begin decomposing at the same temperature (around 270 °C), HPMC undergoes a more rapid weight loss than zein. TFV experiences some weight loss at around 320 °C, but after this decomposition step it still maintains over 60% of its initial weight. These results match previous TGA profiles in the literature (Notario-Pérez et al., 2018; Gupta et al., 2016; Brahatheeswaran et al., 2012). A weight loss of around 70–90 °C is observed for these three materials, which may be due to the loss of the water captured by these products during storage. This behaviour is observed in only polymers and drugs as they are powdery materials and are more affected by environmental humidity. All plasticisers show similar behaviour with almost complete decomposition between 200 °C and 310 °C.

It can be seen in Fig. 4A and B that HPMC films without plasticiser (H100 and H200) have a similar profile to HPMC raw material, but at the end of the test they still retain around 30% of their weight due to the presence of TFV. A double decomposition is observed in films including glycerol as a plasticiser: the first at around 170 °C, corresponding to glycerol, and a second at around 270 °C for HPMC. However, HPMC films that include PEG as a plasticiser demonstrate interesting behaviour. Although the profile could be expected to be

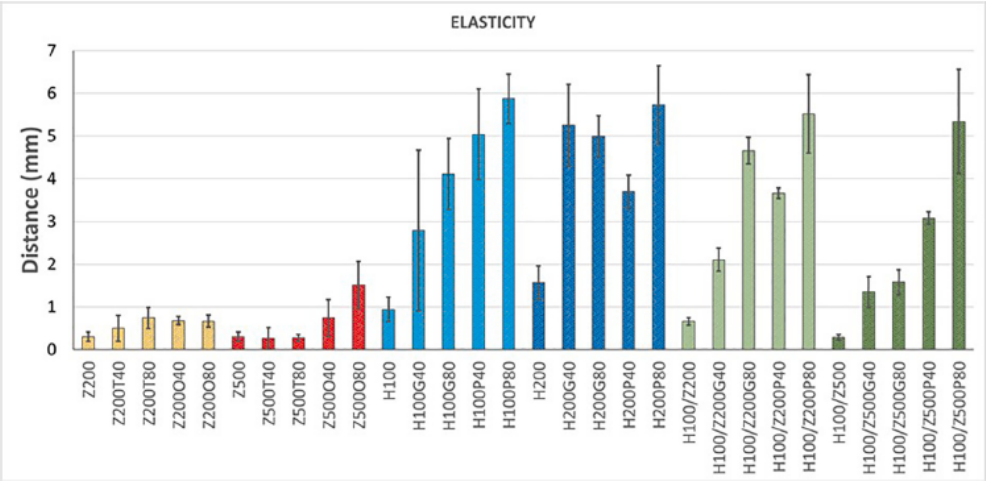

Fig. 2. Results of the films' elasticity, expressed as the distance (mm) they are deformed before breaking.

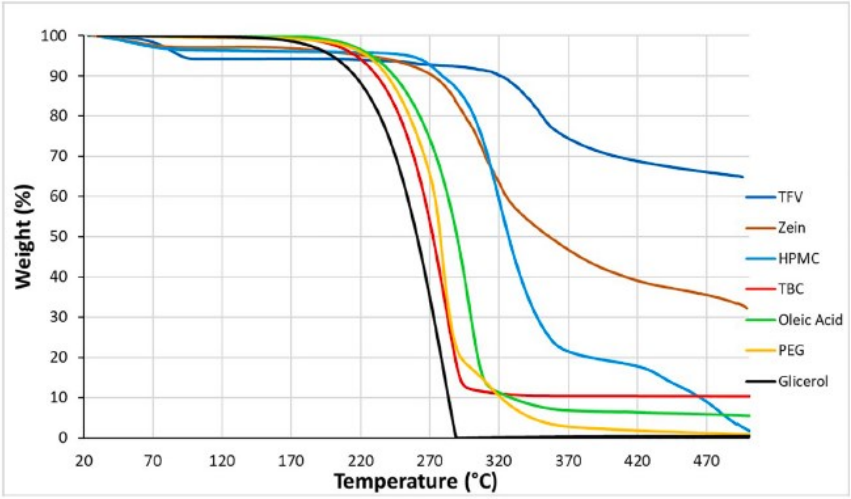

Fig. 3. TGA curves of raw materials used to prepare the films.

similar to films with glycerol, the test reveals a similar TGA profile to batches without plasticiser. This is related to a higher polymer-plasticiser interaction which overrides the characteristic decomposition curve of PEG and causes them to decompose at the same time. The increased interaction of HPMC with PEG compared to glycerol is related to the greater interface in the mixture, meaning better dispersion and compatibility, as already described in polymer blends (Zhang et al., 2018).

The TGA curve of zein films without plasticiser (Fig. 4C and D) is again similar to zein raw material. Zein-based films containing plasticisers undergo only one decomposition rather than a double weight loss, which is observed at around 250 °C. This again indicates a stronger

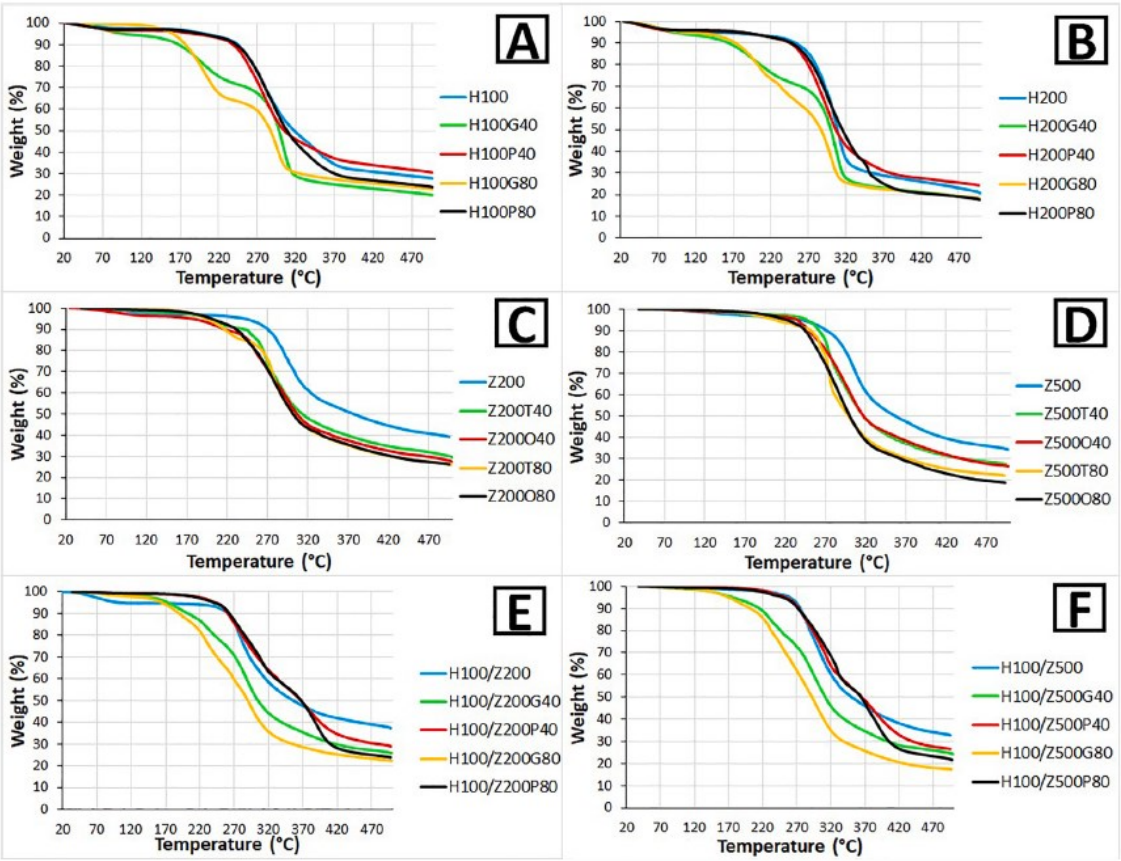

Fig. 4. TGA curves of films with 100 mg of HPMC (A), 200 mg of HPMC (B), 200 mg of zein (C), 500 mg of zein (D), 100 mg of HPMC with 200 mg of zein (E), and 100 mg of HPMC with 500 mg of zein (F).

interaction with the plasticiser. It should be noted that while all the films in batches with 200 mg of zein have a similar profile, differences can be seen in batches with 500 mg of zein when the amount of plasticiser is higher (batches Z200T80 and Z200O80), and the final weight is lower. Although previous works have studied the incorporation of up to 100% of these plasticisers (w/w of zein) (Zhang et al., 2015), this TGA profile suggests that the amount of plasticiser accepted by the polymer has been exceeded, and that the overload not captured in the films has decomposed independently. This also matches the observation in Table 2, where these films are described as "very greasy".

Finally, the same behaviour described for films with HPMC as a single polymer is observed for mixed HPMC/zein films (Fig. 4E and F). That is, while in films containing glycerol as a plasticiser the decomposition of the plasticiser begins around 170 °C and is then followed by the decomposition of the polymers, films with PEG form a mixed film and both polymer and plasticiser decompose at the same time, revealing a good interaction between HPMC, zein and PEG.

We can therefore expect better results from batches including PEG than from films with glycerol, since the polymer-plasticiser interaction is stronger. This agrees with the films' characteristics observed in pliability results (Table 2), where mixed HPMC/zein batches including glycerol are mainly described as "flexible", while those batches that include PEG as plasticiser are mainly described as "completely flexible". Thus, the stronger interaction offered by PEG gives films more flexibility.

3.5. Bioadhesion test

One of the most important properties required by films is bioadhesion, since effective prophylaxis is only possible if the formulation is retained at the target site (Caramella et al., 2015). To conduct a preliminary screening of the formulations, a bioadhesion residence time test was performed using tanned goat hide as a substrate, which has been shown to be a good model for bioadhesion evaluation (Campaña-Seoane et al., 2014) and has been used previously to evaluate vaginal formulations (Martín-Illana et al., 2018).

The outcomes of the bioadhesion study were excellent. Films containing only HPMC as the polymer remained attached to the biological sample until their complete erosion. This was predictable, as HPMC's adhesive properties, thanks to its hydrogen bonds, have been extensively studied (Notario-Pérez et al., 2017; Odeniyi et al., 2015). Films with zein and films combining HPMC and zein remained attached to the vaginal mucosa for at least ten days, when the trial was ended. Zein's adhesive properties are also previously referenced in the literature (Notario-Pérez et al., 2018; Alqahtani et al., 2017). This therefore ensures that the drug will be completely released before the formulation detaches.

All the films are therefore valuable in terms of bioadhesion for further trials that confirm their *in vivo* ability to release the drug in a sustained way.

3.6. Film behaviour in simulated vaginal fluid

The evaluation of the films' behaviour in SVF provides information on the swelling and erosion of the film in the vaginal environment, which may be related with the release of the drug.

Although the films with 100 mg of HPMC (Fig. 5A) all have similar swelling profiles, the batch of films without plasticiser (H100) is observed to capture most water (around 3100% w/w), followed by films with PEG and then those with glycerol. However, the swelling and erosion profiles of all these batches are very similar. This pronounced swelling ability of HPMC has already been demonstrated (Notario-Pérez et al., 2017; Bartkowiak et al., 2018). Nor is there any significant difference between batches with 200 mg of HPMC (Fig. 5B), where once again the batches with glycerol capture the least water; this is probably because glycerol does not interact properly with the HPMC chains, as already observed in the TGA curves (Fig. 4A). In contrast, films with PEG show a more similar behaviour to films without plasticiser, which may be due to the stronger interaction between HPMC and PEG described previously.

Zein films are significant, as water capture is lower (Wang et al., 2017) and complete erosion is never achieved (Fig. 5C and D). This was to be expected as this polymer is unable to form a gel in the presence of water but can still capture some water, which is incorporated in its structure and leads to the relaxation of the zein polymer chains (Berardi et al., 2018). This explains why there is a rapid initial weight gain in the water capture profile, which then remains constant and is not eroded; the water does not swell the zein, but is incorporated into the film's structure and acts as a plasticiser. However, one unexpected result of our test is that although the water capture is normalized according to the amount of polymer (by Eq. (2)), batches with the same amount of zein do not have the same residue after the experiment. Thus, among batches with 200 mg of zein (Fig. 5C), the films without plasticiser are those with the lowest final residue. The reason for this behaviour is that zein films are so rigid that they allow practically no water incorporation. Plasticisers increase the flexibility of the polymer chains and therefore admit more water, which is strongly incorporated into the polymer structure, confirming the interaction observed in the thermal analysis of the films. Z200 is the batch with the lowest water capture (up to around 50% of AWC), while batches with oleic acid capture up to 80%, and those with TBC up to 110–120% of AWC. Batches with 500 mg of zein (Fig. 5D) incorporate more water since they are thicker. Batch Z500 and batches with TBC have a very similar behaviour in terms of water capture, which is to be expected since this plasticiser does not modify the films' flexibility (Fig. 2). However, films with 500 mg of zein and oleic acid had a lower final residue, clearly indicating that some of the oleic acid included in the film had dissolved (the more oleic acid was included, the less final undissolved residue remained). This peculiar behaviour, which was explained by Gezer et al., is due to the orientation of the carboxylic groups of the oleic acid towards the interface with zein, thus enhancing its hydrophilic character (Gezer et al., 2015).

Finally, all batches of mixed HPMC/zein films show similar profiles in vaginal fluid, which is in fact an intermediate behaviour between HPMC and zein films (Fig. 5E and F). The maximum water capture of mixed films is between that of the films containing only one polymer; this behaviour is similar to the previous combinations of zein with hydrophilic polymers (Wang et al., 2017). The final undissolved residue is lower than in pure zein films, although there is still some remnant; this is lower in batches with only 200 mg of zein as the HPMC and plasticisers are always eroded. Lastly, it can be seen that the batches with glycerol have lower water capture and less final residue than those with PEG, revealing again the stronger interaction between PEG and the polymers.

3.7. Release study

The profile of the TFV released from the films can be seen in Fig. 6. HPMC films are unable to control TFV release over long periods; all the batches with 100 mg of HPMC are very similar, and the complete release of the drug is achieved at 24 h (Fig. 6A). This behaviour is similar to vaginal gels, which are designed for daily application (Bunge et al., 2018; Beigi et al., 2016). This is to be expected since, as can be seen in the previous test, the swelling of the films occurs in a few minutes and the films are transformed into a HPMC gel that is able to control TFV release (Notario-Pérez et al., 2017; Akhlaq et al., 2018). Somewhat more surprising is the behaviour of films including 200 mg of HPMC (Fig. 6B). Although usually the more HPMC the formulation contains, the more sustained the release (Notario-Pérez et al., 2018), these films deliver all the TFV in 12 h, considerably faster than films with half the amount of swellable polymer. The explanation for this faster release can be found in the appearance and structure of these films, because (as

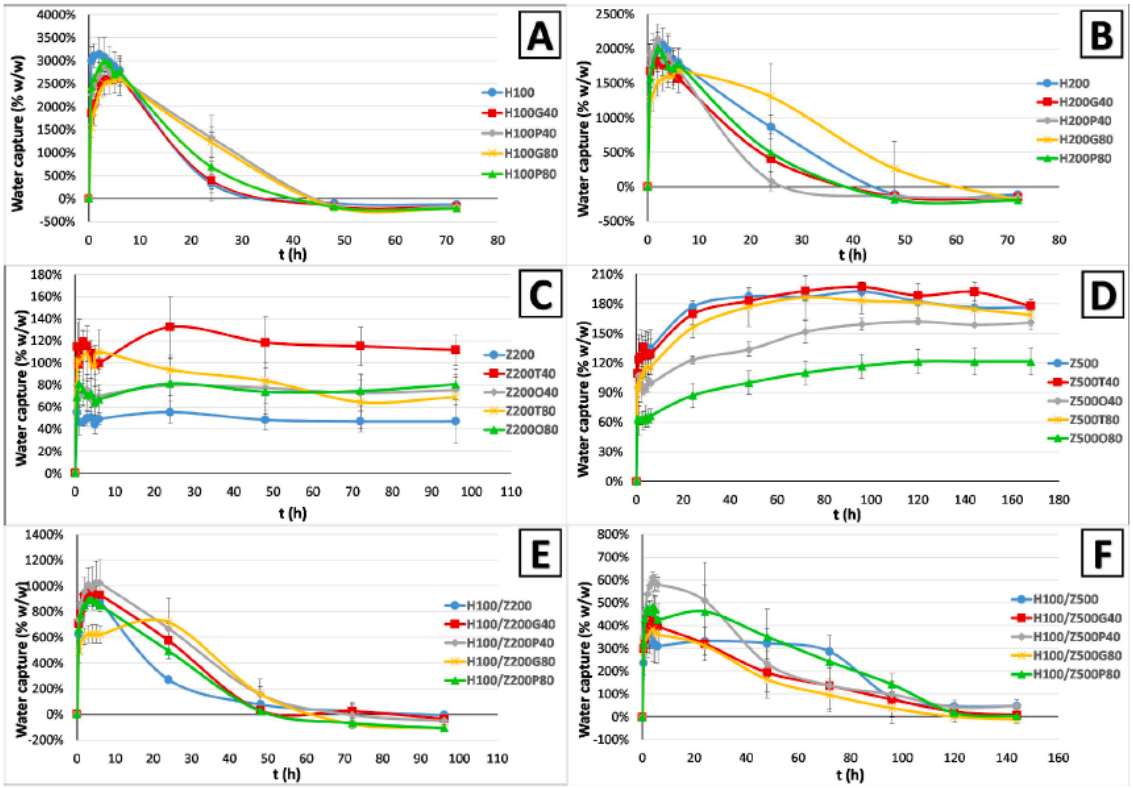

Fig. 5. Adjusted water capture of films with 100 mg of HPMC (A), 200 mg of HPMC (B), 200 mg of zein (C), 500 mg of zein (D), 100 mg of HPMC with 200 mg of zein (E), and 100 mg of HPMC with 500 mg of zein (F).

described in Table 2) when films are prepared with 200 mg of HPMC they are not completely uniform but have some bubbles (and even holes). This means that once in the vaginal environment, instead of becoming a homogeneous gel layer, they form a gel with surface pores in their structure that make drug diffusion easier. This behaviour can also be seen in Fig. 5, since the percentage of water captured by H200 batches is much lower than for H100 batches. The effect of the plasticiser can also be seen in these films, and the H200P40 batch is able to achieve a slightly more sustained release of the drug. However, it is evident that the objective of TFV release over several days cannot be achieved by films including only HPMC.

Zein shows greater potential for controlled-release films, since the amount of polymer in the films substantially conditions the release of TFV. The Z200 batch does not represent any improvement on HPMC films in terms of drug release control, as 90% of the TFV is released in only four hours (Fig. 6C). However, batch Z500 achieves a controlled release over 48 h (Fig. 6D). Zein films show a marked effect of the plasticiser on the control of drug release. Oleic acid, due to the ability of the zein-oleic acid mixture to self-assemble through hydrophobic-hydrophilic interactions (Wang et al., 2017; Lai et al., 1999), produces different layers of oleic acid and zein that significantly hinder the release of TFV. Batch Z200O80 and Z500O80 are able to release TFV in a sustained manner over 72 h and 84 h respectively, notably improving the control offered by films without a plasticiser. In contrast, zein batches with TBC as a plasticiser barely modify the behaviour of zein.

Lastly, the control of TFV release by films combining HPMC and zein is analysed. Although we could expect to see an intermediate behaviour between the control provided by the polymers alone, this was not the case. The batch combining HPMC and zein in a ratio of 1:2 (batch H100/Z200) had poorer control over the release than H100 and Z200 batches; the release profile shows that 80% of the drug is delivered in only three hours (Fig. 6E). Nor are any differences observed when the plasticisers are included. The explanation for this behaviour is clear: at this ratio, zein does not have the structure to control the release of TFV, and HPMC cannot achieve a strong enough gel layer to hinder the diffusion of the drug. The combination of HPMC/zein in a ratio of 1:5 (batch H100/Z500) successfully controls release for 24 h, but does not improve the outcomes of batches HPMC100 and Z500. Another unexpected result was observed when PEG was included in these films. As was seen in H200 films, the inclusion of a small amount of PEG (40% w/w) can modify the release of TFV. Thus, batch H100/Z500P40 stands out from the others with a sustained release profile of TFV of 120 h. PEG acts as a plasticiser in its structure, and possibly also as a compatibilizer between the polymers, improving the interface area (Zhang et al., 2018; Zhang et al., 2013). The presence of PEG is therefore indispensable to develop mixed HPMC-zein films. With this outcome, these films are an excellent option for further clinical trials, since their application would offer women protection for five days. TFV inhibitory concentration 50 (IC_{50}), which is achieved at 1.08–1.22 μM (Notario-Pérez et al., 2017), is reached quickly after the administration of the film and it is maintained while drug is being released.

Finally, it is also worth noting that the control is poorer when more PEG is included (batch H100/Z500P80), as was also observed in the H200P80 batch. An excess of plasticiser was therefore added to these batches, and as PEG is a hydrophilic molecule, the amount not incorporated in the polymer structure dissolves in the presence of vaginal fluid and generates pores which facilitate TFV diffusion. Thus not only the presence but also the amount of this plasticiser is critical in designing an optimal formulation.

All the release profiles in Fig. 6 were fitted to different model-dependent kinetics (zero order, Higuchi, Hopfenberg and Ritger-Peppas). In almost all batches containing 100 mg of HPMC, the Ritger-Peppas *n* value is lower than 0.5, indicating a pure diffusion mechanism. This was predictable, because as observed in the swelling test, the films very

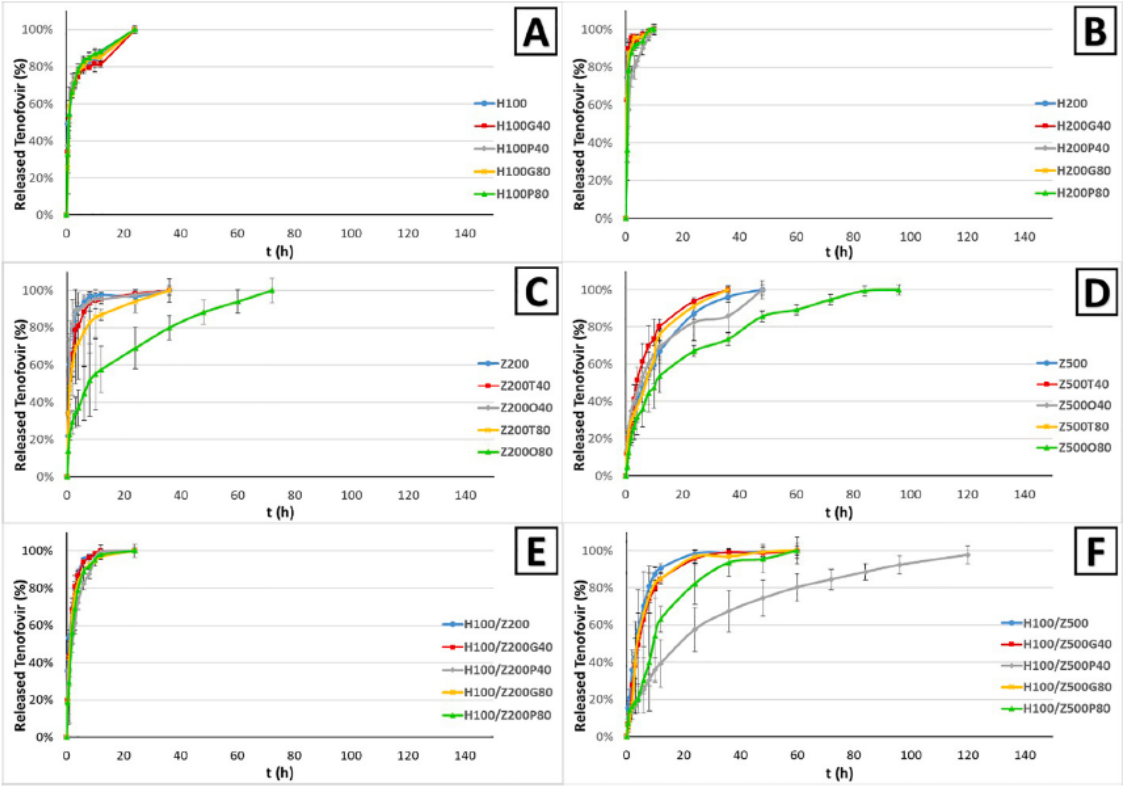

Fig. 6. TFV release profiles of films with 100 mg of HPMC (A), 200 mg of HPMC (B), 200 mg of zein (C), 500 mg of zein (D), 100 mg of HPMC with 200 mg of zein (E), and 100 mg of HPMC with 500 mg of zein (F).

quickly form a gel and all the drug is released before the onset of erosion. Only batch H100G80 has a higher value of n (0.68), which points to a combination of film erosion and drug diffusion. However, films from this batch do not enhance the control over TFV release, so the most probable explanation for this phenomenon is not an erosion of the HPMC, but rather that the excess glycerol dissolves in the vaginal fluid. This hypothesis is supported by the relatively good fit of this batch to the Higuchi model, which confirms that diffusion is the predominant drug release mechanism. Batches with 200 mg of HPMC clearly differentiate the TFV release mechanism according to the plasticiser included; batches without plasticiser or with glycerol have very low values of n when they fit the Ritger-Peppas model, and also have an excellent fit to the Higuchi model, so diffusion of TFV through HPMC is clearly the drug release mechanism in these batches. However, batches with PEG (H200P40 and H200P80) have higher n values for Ritger-Peppas, indicating a combination of drug diffusion and polymer erosion. As observed in Fig. 6, these batches prolong TFV release, so the gel begins to erode after all the drug is released.

All batches that include only zein as a polymer have an excellent fit to the Higuchi model, so diffusion through the polymer matrix is the predominant drug release mechanism. Nevertheless, some of these batches also have a value of n in the Ritger-Peppas model which suggests a combination of drug diffusion and matrix erosion. This again agrees with the results obtained in the evaluation of the films' behaviour in simulated vaginal fluid; although zein is not completely eroded, it modifies its structure in the vaginal environment.

Finally, batches combining HPMC and zein have a good fit to both Higuchi and Hopfenberg, indicating that drug diffusion and polymer erosion are more clearly present in these films, which was expected since HPMC swells and erodes in the presence of SFV, although zein never erodes completely. Values of n in the adjustment to the Ritger-Peppas model are mainly between 0.5 and 1, supporting this conclusion. However, two batches are worth highlighting: on the one hand, batch H100/Z500G80 has an n value of 1.26, which implies the structural modification of the polymer matrix; and on the other, batch H100/Z500P40, which shows the most sustained TFV release, has an n value of 0.49, suggesting the predominance of drug diffusion. This again confirms this as being the optimal combination, since it can be transformed into a robust and homogeneous film where none of the components erodes faster than the others.

The values of the similarity factor (f_2) were calculated in order to statistically demonstrate the differences between batches. Briefly, it can be seen that there are no differences between batches with 100 mg of HPMC. In batches manufactured with zein as a single polymer, those containing 80% oleic acid (% w/w) are substantially different from the other films, thus confirming the improvement observed in Fig. 6. Finally, in the combinations of HPMC and zein, significant differences can be observed when batches containing PEG as a plasticiser are compared with the others. This is undoubtedly also related with the different drug release mechanism of these batches as mentioned previously. The improved drug release obtained in batch H100/Z500P40 is therefore statistically demonstrated.

Appendix A shows all the values obtained for the fit of each batch to the different kinetics (Tables A1–A3), as well as the values of f_2 comparison (Table A4).

3.8. Cytotoxicity assessment

To study the cell toxicity, the materials were incubated in PBS 1x at room temperature for 48 h before the assay to ensure that any potential toxic component would be present in the suspension to be tested. The cell culture was then treated with this suspension at different

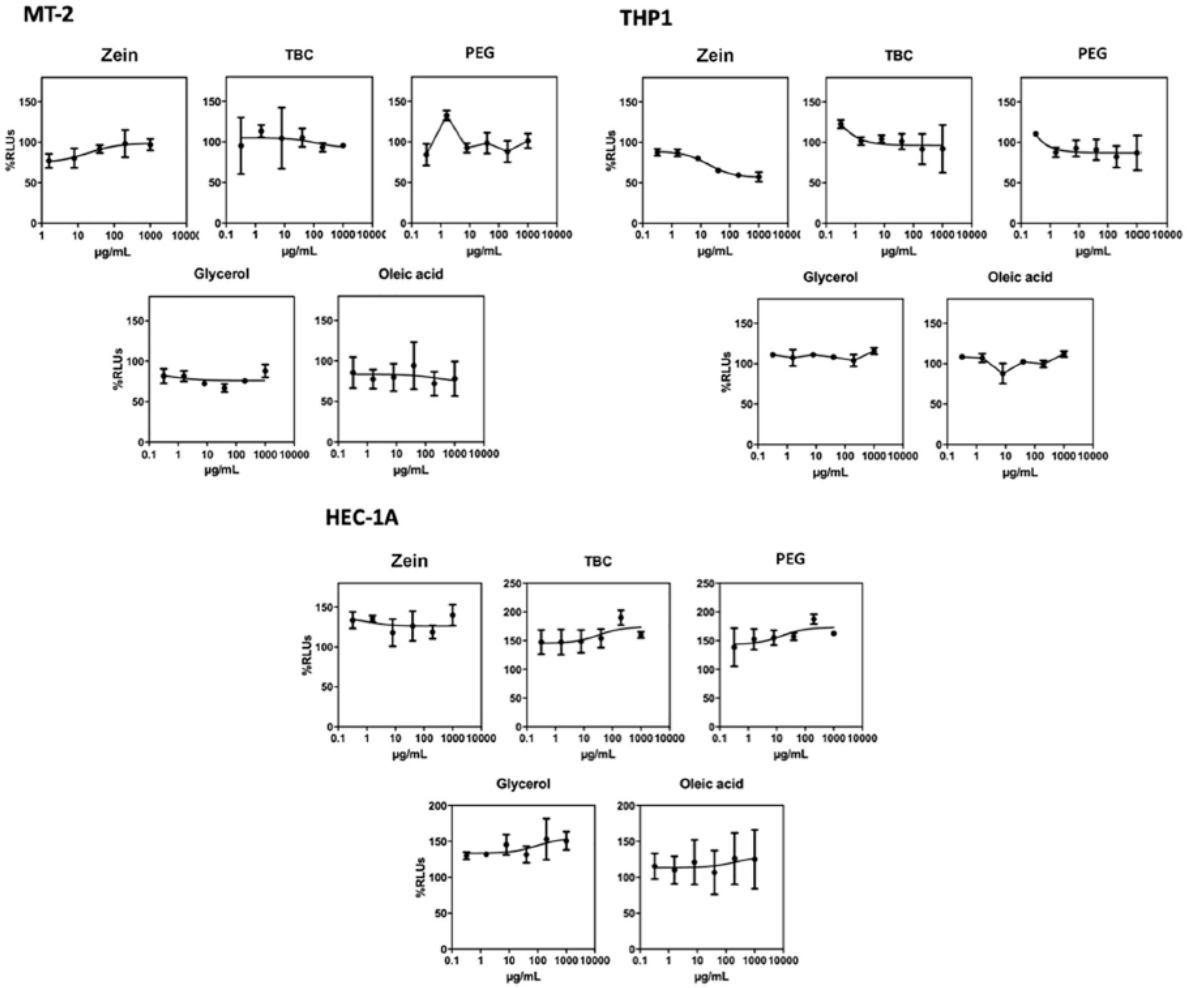

Fig. 7. Graphic representation of the cytotoxic evaluation of the film materials zein, glycerol, PEG, TBC and oleic acid in MT-2, THP1 and HEC-1A cells. Cell viability is expressed as percentage of living cells (%RLUs) as compared to a non-treated control (1 0 0).

concentrations at a maximum concentration of 1000 µg/mL.

Experiments were performed in lymphoblastic (MT-2) and macrophage-monocyte-derived cell lines to evaluate toxicity on the immune cells present in vaginal or cervical mucosae, and in a cervical epithelial cell line (HEC-1A) to assess the potential damage to the integrity of the mucosae (Fig. 7).

As shown in Fig. 7 and Table 3, all the materials tested were biocompatible in the three cell types, displaying CC_{50} values greater than 1000 µg/mL. Only zein showed a slight tendency to induce cell toxicity at the maximum concentration tested (1000 µg/mL) in THP1 cells, but not enough to calculate the CC_{50} value.

Table 3
Results of the cytotoxicity analysis of the materials tested in MT-2, THP-1 and HEC-1A cells. Cytotoxic concentrations 50 (CC_{50}) were calculated using GraphPad Prism software.

CC_{50} µg/mL	MT-2	THP1	HEC-1A
Zein	> 1000	> 1000	> 1000
TBC	> 1000	> 1000	> 1000
PEG	> 1000	> 1000	> 1000
Glycerol	> 1000	> 1000	> 1000
Oleic acid	> 1000	> 1000	> 1000

4. Conclusions

The combination of HPMC and zein in vaginal films produces a robust formulation that enhances the characteristic properties of both polymers. Sustained release of TFV is possible thanks to the amphiphilic nature of zein –which hinders drug diffusion– and the inclusion of a small amount of HPMC, which gives the film excellent mechanical properties. Both polymers have demonstrated bioadhesive attributes that ensure the retention of the film in the vaginal environment.

The inclusion of a plasticiser clearly modifies the behaviour of the films by improving both their flexibility and their water permeability, which will determine the drug release. Thus the inclusion of PEG (40% w/w) to mixed HPMC/zein (ratio 1:5) films achieves a completely flexible and bioadhesive formulation which is able to release TFV in a sustained way for 120 h. This formulation is therefore highly valuable for subsequent clinical trials, since its application could protect women from sexual acquisition of HIV for up to five days after its administration.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Author contributions

Fernando Notario-Pérez, Roberto Ruiz-Caro, Luis Miguel Bedoya and María-Dolores Veiga designed and planned the experiments. Fernando Notario-Pérez, Araceli Martín-Illana, Raúl Cazorla-Luna, Luis Miguel Bedoya and Juan Peña conducted the experiments. All the authors have approved the final article. María-Dolores Veiga is the senior author and project leader.

Appendix A

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Table A1
Correlation coefficients obtained when experimental data from batches manufactured with HPMC as a single polymer are fitted to the different mathematical models.

Batch	Zero order		Higuchi		Hopfenberg		Ritger-Peppas		
	r^2	K_0	r^2	K_H	r^2	K_{HF}	r^2	K_{RP}	n
HPMC100	0.5367	0.055	0.7942	0.229	0.6550	0.044	0.9397	0.56	0.22
HPMC100 G40	0.5367	0.046	0.7897	0.209	0.6303	0.035	0.9754	0.49	0.48
HPMC100 G80	0.6284	0.084	0.8609	0.295	0.7264	0.064	0.8340	0.45	0.68
HPMC100 PEG40	0.6273	0.082	0.8736	0.290	0.7286	0.063	0.9949	0.50	0.46
HPMC100 PEG80	0.6507	0.084	0.8887	0.295	0.7592	0.759	0.9404	0.49	0.50
HPMC200	0.8753	0.901	0.9842	0.929	0.9384	0.686	0.7771	0.84	0.14
HPMC200 G40	0.9511	0.896	0.9999	0.894	0.9934	0.678	0.8593	0.81	0.30
HPMC200 G80	0.9305	0.875	0.9990	0.882	0.9798	0.647	0.8139	0.79	0.24
HPMC200 PEG40	0.7825	0.190	0.9474	0.441	0.8538	0.141	0.9445	0.50	0.67
HPMC200 PEG80	0.9979	0.783	0.9236	0.733	0.9796	0.535	0.8449	0.62	0.64

Table A2
Correlation coefficients obtained when experimental data from batches manufactured with zein as a single polymer are fitted to the different mathematical models.

Batch	Zero order		Higuchi		Hopfenberg		Ritger-Peppas		
	r^2	K_0	r^2	K_H	r^2	K_{HF}	r^2	K_{RP}	n
Z200	0.8322	0.336	0.9034	0.503	0.8482	0.216	0.8134	0.44	0.79
Z200 TBC40	0.8198	0.181	0.9765	0.417	0.8952	0.133	0.9715	0.48	0.47
Z200 TBC80	0.6903	0.081	0.9160	0.282	0.7942	0.061	0.9927	0.46	0.40
Z200 AO40	0.8331	0.404	0.9413	0.616	0.8744	0.290	0.8414	0.59	0.62
Z200 A080	0.7579	0.018	0.9544	0.129	0.8513	0.013	0.9854	0.20	0.42
Z500	0.9083	0.048	0.9974	0.189	0.9479	0.031	0.9909	0.20	0.48
Z500 TBC40	0.9042	0.063	0.9871	0.247	0.9532	0.044	0.9927	0.19	0.67
Z500 TBC80	0.9427	0.053	0.9812	0.203	0.9653	0.035	0.9723	0.19	0.49
Z500 AO40	0.7602	0.030	0.9636	0.172	0.8572	0.022	0.9952	0.24	0.43
Z500 AO80	0.7938	0.019	0.9662	0.132	0.8638	0.013	0.9547	0.10	0.70

Table A3
Correlation coefficients obtained when experimental data from batches manufactured with a combination of HPMC and zein are fitted to the different mathematical models.

Batch	Zero order		Higuchi		Hopfenberg		Ritger-Peppas		
	r^2	K_0	r^2	K_H	r^2	K_{HF}	r^2	K_{RP}	n
HPMC100/Z200	0.8521	0.240	0.9909	0.466	0.9240	0.171	0.9843	0.50	0.47
HPMC100/Z200 G40	0.9473	0.270	0.9677	0.492	0.9822	0.191	0.9768	0.38	0.90
HPMC100/Z200 G80	0.9038	0.202	0.9783	0.445	0.9550	0.147	0.9738	0.38	0.83
HPMC100/Z200 PEG40	0.8917	0.131	0.9889	0.355	0.9533	0.094	0.9663	0.31	0.67
HPMC100/Z200 PEG80	0.9560	0.195	0.9759	0.416	0.9855	0.135	0.9894	0.31	0.78
HPMC100/Z500	0.9519	0.096	0.9869	0.296	0.9865	0.068	0.9841	0.22	0.62
HPMC100/Z500 G40	0.9314	0.072	0.9775	0.276	0.9755	0.052	0.9902	0.12	0.94
HPMC100/Z500 G80	0.9121	0.076	0.9501	0.292	0.9582	0.055	0.9869	0.08	1.26
HPMC100/Z500 PEG40	0.9009	0.011	0.9906	0.104	0.9531	0.008	0.9671	0.10	0.49
HPMC100/Z500 PEG80	0.9268	0.034	0.9525	0.178	0.9663	0.024	0.9389	0.11	0.61

Table A4
Similarity factor (f_2) values for the release profiles obtained from reference and test formulations. Comparisons with significant difference ($f_2 < 50$) are in bold.

Reference	Test	f_2	Reference	Test	f_2	Reference	Test	f_2
HPMC100	HPMC100 G40	62.8	Z200	Z200 TBC40	58.7	HPMC100/Z200	HPMC100/Z200 G40	60.6
HPMC100	HPMC100 PEG40	67.4	Z200	Z200 AO40	55.3	**HPMC100/Z200**	**HPMC100/Z200 PEG40**	**43.4**
HPMC100	HPMC100 G80	55.0	**Z200**	**Z200 TBC80**	**43.9**	HPMC100/Z200	HPMC100/Z200 G80	57.7
HPMC100	HPMC100 PEG80	62.9	**Z200**	**Z200 AO80**	**20.8**	**HPMC100/Z200**	**HPMC100/Z200 PEG80**	**47.2**
HPMC100 G40	HPMC100 PEG40	70.9	Z200 TBC40	Z200 AO40	52.5	**HPMC100/Z200 G40**	**HPMC100/Z200 PEG40**	**47.8**
HPMC100 G40	HPMC100 G80	68.2	Z200 TBC40	Z200 TBC80	55.6	HPMC100/Z200 G40	HPMC100/Z200 G80	72.6
HPMC100 PEG40	HPMC100 PEG80	76.5	**Z200 AO40**	**Z200 AO80**	**19.5**	HPMC100/Z200 PEG40	HPMC100/Z200 PEG80	65.0
HPMC100 G80	HPMC100 PEG80	74.0	**Z200 TBC80**	**Z200 AO80**	**27.7**	HPMC100/Z200 G80	HPMC100/Z200 PEG80	62.0
HPMC200	HPMC200 G40	64.5	**Z500**	**Z500 TBC40**	**50.0**	HPMC100/Z500	HPMC100/Z500 G40	57.3
HPMC200	**HPMC200 PEG40**	**33.5**	Z500	Z500 AO40	65.9	**HPMC100/Z500**	**HPMC100/Z500 PEG40**	**22.5**
HPMC200	HPMC200 G80	68.3	Z500	Z500 TBC80	73.0	HPMC100/Z500	HPMC100/Z500 G80	53.0
HPMC200	**HPMC200 PEG80**	**42.1**	**Z500**	**Z500 A080**	**44.9**	**HPMC100/Z500**	**HPMC100/Z500 PEG80**	**30.5**
HPMC200 G40	**HPMC200 PEG40**	**35.7**	Z500 TBC40	Z500 AO40	53.9	**HPMC100/Z500 G40**	**HPMC100/Z500 PEG40**	**25.9**
HPMC200 G40	**HPMC200 G80**	80.2	Z500 TBC40	Z500 TBC80	50.4	HPMC100/Z500 G40	HPMC100/Z500 G80	68.9
HPMC200 PEG40	**HPMC200 PEG80**	**48.8**	**Z500 AO40**	**Z500 AO80**	**42.5**	**HPMC100/Z500 PEG40**	**HPMC100/Z500 PEG80**	**41.7**
HPMC200 G80	**HPMC200 PEG80**	**48.1**	**Z500 TBC80**	**Z500 AO80**	**41.9**	**HPMC100/Z500 G80**	**HPMC100/Z500 PEG80**	**34.2**

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CAPÍTULO VIII

INFLUENCE OF PLASTICISERS ON pH-DEPENDENT DRUG RELEASE AND CELLULAR INTERACTIONS OF HYDROXYPROPYL METHYLCELLULOSE/ZEIN VAGINAL ANTI-HIV FILMS CONTAINING TENOFOVIR

Influence of plasticisers on pH-dependent drug release and cellular interactions of hydroxypropyl methylcellulose/zein vaginal anti-HIV films containing tenofovir

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ABSTRACT

The development of "smart" microbicides that could increase the vaginal release of antiretroviral drugs following intercourse could be an interesting strategy for the prevention of male-to-female sexual transmission of HIV. This work was aimed at developing vaginal films that featured pH-dependent release of tenofovir (TFV), a nucleotide reverse transcriptase inhibitor. Previously developed films, based on hydroxypropyl methylcellulose (HPMC) and zein, were prepared in this work incorporating different plasticisers (oleic acid, lactic acid, glycerol and polyethylene glycol 400 (PEG)) and evaluated for *in vitro* drug release in an acidic simulated vaginal fluid (pH 4.2) and a slightly alkaline mixture of simulated seminal and vaginal fluids (pH 7.5). Results revealed that optimal biphasic release of TFV was possible with proper combination of plasticisers and tuning of plasticiser/matrix-forming materials ratio in films. In particular, PEG and oleic acid (1:7 w/w) comprising 80% (w/w) the amount of film-forming ingredients featured the highest increase in TFV release rate when the pH of simulated vaginal fluid was neutralised by seminal fluid. Furthermore, these films resulted in similar levels of TFV associated with genital epithelial cells, but lower drug permeability as compared with free TFV, when tested in HEC-1-A or Ca Ski cell monolayer models. These data support that films have the potential to allow reaching suitable mucosal levels of TFV with low systemic exposure. Overall, the optimised formulation could be useful in protecting women from sexual transmission of HIV.

1. INTRODUCTION

Great advances have been achieved in recent years towards the goal of ending the HIV/AIDS pandemic, especially in terms of access to antiretroviral therapy and implementation of new preventative strategies (e.g. oral pre-exposure prophylaxis) [1]. However, significant gaps remain, affecting in particular more vulnerable population groups. For instance, women in sub-Saharan Africa are exposed to sexual transmission at younger ages than men, accounting for almost 80% of new cases in the 10-19 years old age group [2]. Among other reasons, younger women often lack of power to negotiate the use of condoms with their male partners due to gender inequalities and cultural issues. Vaginal microbicides that can prevent early viral transmission events at the mucosal level and may be used without the consent of men stand as an interesting approach to tackle such gap [3].

Tenofovir (TFV) is a nucleotide reverse transcriptase inhibitor and a long-standing candidate for vaginal microbicide development due to its potency, prolonged half-life and safety profile [4]. The drug was demonstrated as partially effective in preventing male-to-female transmission in a phase 2b clinical trial testing a TFV 1% vaginal gel [5]. Lack of consistent use by women was implicated in poor protection, and later on correlated with reduced mucosal levels of the drug [6]. Adherence problems were confirmed in subsequent trials testing similar TFV gels, which did not account for significant efficacy when compared to placebo products [7, 8]. The coitally-dependent nature of used gels requires that these products are administered close to the time of intercourse in order to sustain protective drug levels at the cervicovaginal mucosa. Alternative strategies based on coitally-independent products that allow sustaining local drug levels have emerged, with particular emphasis on rings [9]. These can be used continuously for weeks/months and release active payloads in a controlled fashion. Still, clinical data on a dapivirine vaginal ring showed only mild efficacy in preventing male-to-female HIV transmission, and that adherence remained an issue [10, 11]. Continuous use of rings was regarded as cumbersome, particularly among younger women. Thus, the microbicides field may benefit of the development of products that could be used on-demand but still allow providing high drug levels at the mucosa upon exposure to the virus. Formulations featuring pH-dependent seem particularly appealing for such purpose. In particular, the well-known acid-to-slightly alkaline pH change observed due to the deposition of semen (the main source of HIV) in the vagina [12] could trigger an increase in drug release that boosts cervicovaginal drug levels. Such systems are often based on polymers featuring differential solubility according to the pH of media [13-15].

Vaginal films are a particularly interesting dosage form for the development of vaginal microbicides [16]. These systems usually comprise thin polymeric sheets combining the advantages of vaginal solid and semisolid dosage forms. For example, films avoid the leakage and messiness associated with gels, while presenting the typical excellent stability of rings and tablets [17]. In addition, films are easy and cheap to manufacture at the industrial scale, and do not require an applicator for administration [18]. Vaginal films based on mixtures of hydroxypropyl methylcellulose (HPMC) and zein have been previously developed for drug sustained release [19]. In particular, it was observed that films produced at a HPMC/zein ratio of 1:5 and plasticised with 40% polyethylene glycol 400 (PEG) were able to sustain the release of TFV in a simulated vaginal fluid (SVF; pH 4.2) for at least 120 h. Even if these materials are widely used for obtaining pharmaceutics and especially useful in the fabrication of sustained release dosage forms [20-23], HPMC and zein do not feature any significant changes at the pH range of interest for “smart” microbicides. Thus, this work is aimed at modifying HPMC/zein films in order to allow the release of TFV in a pH-dependent biphasic fashion. Different common film plasticisers were screened, including neutral (glycerol and PEG) and acidic (lactic and oleic

acid) ones, and tested their ability to confer differential erosion/drug release features to films at acidic and slightly alkaline pH. Optimised films were further tested for cytotoxicity, as well as mucosal permeability and retention of TFV using relevant cell-based models for vaginal drug delivery.

2. MATERIALS AND METHODS

2.1. Materials

HPMC (Methocel® K 100 M) was kindly provided by Colorcon Ltd. (Kent, UK). Zein, oleic acid, PEG 400 (Kollisolv® PEG E 400) rat tail collagen type I and resazurin were purchased from Sigma-Aldrich (St. Louis, MO, USA). L-(+)-lactic acid and glycerol were acquired from Panreac (Barcelona, Spain). TFV was supplied by Carbosynth Limited (Berkshire, UK). McCoy's 5A medium was acquired from Alfagene (Carcavelos, Portugal), RPMI 1640 medium and Hank's balanced salt solution (HBSS) from Gibco (ThermoFisher Scientific, Waltham, MA, USA) and Dulbecco's Modified Eagle medium (DMEM) from Lonza (Verviers, Belgium). Penicillin, streptomycin and fetal bovine serum were purchased from Invitrogen (ThermoFisher Scientific, Waltham, MA, USA). Methanol and all other reagents were of analytical grade and used without further purification. Demineralised water was used in all cases.

2.2. Preparation of films

Films were obtained using a solvent casting method previously described [19]. Matrix-forming materials (100 mg of HPMC and 500 mg of zein) and the drug were placed into individual silicone templates with 43 mm in diameter, and 10 mL of a methanol/water solution of plasticisers was added and gently stirred in order to achieve polymer and protein dissolution/suspension. The templates were maintained at room temperature until complete solvent evaporation.

Table 1. Composition of films prepared. The amount of each ingredient is presented in mg per film.

Film	HPMC (H)	Zein (Z)	PEG	Glycerol (G)	Lactic acid (LA)	Oleic acid (OA)	TFV
HZ-PEG	100	500	240				30
HZ-G	100	500		240			30
HZ-LA	100	500			240		30
HZ-OA	100	500				240	30
HZ-G/LA	100	500		120	120		30
HZ-G/OA	100	500		120		120	30
HZ-PEG/LA	100	500	120		120		30
HZ-PEG/OA	100	500	120			120	30
HZ-PEG1/OA7	100	500	30			210	30
HZ-PEG1/OA3	100	500	60			180	30
HZ-PEG3/OA1	100	500	180			60	30
HZ-PEG1/OA7-20	100	500	15			105	30
HZ-PEG1/OA7-60	100	500	45			315	30
HZ-PEG1/OA7-80	100	500	60			420	30

Screening of different film compositions was conducted in a phased manner. First, films were prepared by including a single plasticiser (glycerol, PEG, lactic acid or oleic acid) and tested for their properties. Then, neutral plasticisers (glycerol and PEG) were combined with the acid ones (lactic and oleic acid) in a 1:1 (w/w) ratio in order to evaluate possible synergies. The best plasticiser combination (PEG and oleic acid) was further tested in different ratios (1:7, 1:3 and

3:1, w/w), as well as at different matrix-forming materials/plasticiser ratios (5:1, 5:2, 5:3 and 5:4, w/w). TFV was incorporated in films at a fixed amount of 30 mg in order to match the dose previously tested in the CAPRISA 004 clinical trial [5]. A summary of tested film formulations is presented in Table 1.

2.3. Technological characterisation of films

2.3.1. Drug release

In vitro drug release tests were performed in a SVF (pH 4.2) [24] and in a mixture of the previous with a simulated seminal fluid (SSF) [25] at a ratio 1:4 (v/v) [26]. The SVF/SSF mixture presented a final pH of 7.5. Testing was carried out following previously described methodology [19]. Films were submerged in 80 mL of pre-heated release medium in screw capped borosilicate glass bottles, and then immediately placed on a shaking water bath at 37 °C and 15 opm. Aliquots (5 mL) were collected periodically and the amount of TFV was quantified by UV spectroscopy (Evolution 60S spectrophotometer, Thermo scientific, Waltham, MA, USA) at 260 nm. Each film was tested in triplicate for both media.

Release profiles were compared by a model independent index, namely the similarity factor (f_2), calculated as follows:

$$f_2 = 50\ log\left\{\left[1 + \left(\frac{1}{n}\right)\sum_{j=1}^{n}\left|R_j - T_j\right|^2\right]^{-0.5}\ x\ 100\right\} \quad \text{(Eq. 1)}$$

where n is the number of included time-points, R_j drug release percentage for the reference formulation and T_j the drug release percentage for the tested one. When f_2 < 50 there are differences among the compared profiles [27].

2.3.2. Swelling behaviour

Swelling and erosion phenomena that films undergo upon immersion in media were evaluated as described by Ruiz-Caro & Veiga-Ochoa [28]. Briefly, films were placed over stainless steel discs and immersed in medium inside a beaker placed on a shaking water bath at 37 °C and 15 opm. Samples were weighed periodically to quantify the swelling ratio percentage (SR). A positive SR implies capture of medium, while negative values indicate the erosion of films. Experiments were performed in triplicate for each film in each media.

2.3.3. Mucoadhesion

The adhesion of films to vaginal mucosa was evaluated by using an *ex vivo* test [29]. Calf vaginal mucosa was obtained from a local slaughterhouse. Tissue samples were immobilised to an 8.5 x 5 cm stainless steel plate by the serosal side with cyanoacrylate adhesive. The film was pressed over the mucosal side for 30 s using a weight of 500 g. The whole system (plate + mucosa + film) was then fully emerged in SVF at an angle of 60° and placed on an orbital shaking water bath at 37 °C and 15 opm. The period required for a film to detach from the mucosa was monitored visually and defined as the mucoadhesion time. Films were tested in duplicate.

2.3.4. Mechanical properties

Mechanical properties of films were evaluated by means of a TA.TXT*plus* Texture Analyser. Methodology is described in the *Supplementary Information* (*S1. Supplementary methods*).

2.3.5. Microscopic morphology

Surface morphology of films was assessed by scanning electron microscopy. Methodology is detailed in the *Supplementary Information* (*S1. Supplementary methods*).

2.4. Cellular studies with films

2.4.1. Cytotoxicity

In vitro toxicity of raw materials and film extracts to human genital cell lines was determined by the resazurin metabolism assay. HeLa cervical, Ca Ski cervical and HEC-1-A endometrial cell lines were obtained from ATCC (Manassas, VA, USA). HeLa, Ca Ski and HEC-1-A cells were maintained in DMEM, RPMI 1640 and McCoy's 5A media, respectively, supplemented with 10% foetal bovine serum, 100 U/mL penicillin and 0.1 µg/mL streptomycin. Media were refreshed every 2-3 days and cells were kept under standard conditions (37 °C, 5% CO_2 and 95% humidity).

Raw materials were tested after dissolution/dispersion in media. Film extracts were prepared by immersing samples in cell culture medium at a film surface-to-medium volume ratio of 1 cm^2/mL in accordance to the ISO 10993-5:2009 standard (Biological evaluation of medical devices - Part 5: Tests for in vitro cytotoxicity). Film samples were extracted for 24 h at 37 °C and 100 rpm, and resulting extracts were collected and tested without dilution (100%) or diluted to 50% and 10% with medium [30].

Cytotoxicity was determined using cells pre-seeded for 24 h under standard conditions in 96-well plates at 5000 cells/well. Film extracts or solutions/suspensions of raw materials in culture media at concentrations in the range of 10 to 0.001 mg/mL were added and cells further incubated for 24 h. Cells were then washed twice with phosphate buffered saline (pH 7.4) and incubated with 1% resazurin (w/v) in medium for 4 h under standard conditions. Fluorescence readings were performed at Ex/Em of 530/590 nm and viability calculated as the percentage of control (cells incubated only with medium). Concentration-dependent viability data were fitted using a log-logistic regression in order to calculate half-maximal cytotoxic concentration (CC_{50}) values. Experiments were performed in triplicate.

2.4.2. *In vitro* permeability and epithelial cell retention

Cell monolayers based on Ca Ski or HEC-1-A cells were obtained as previously described [31, 32]. Cells were seeded at a density of 3 x 10^5 per square centimetre over Millicell® cell culture inserts (Merck-millipore, Billerica, MA, USA). Inserts featured 1.1 cm^2 total area and 1 µm pore, and were pre-coated with rat tail collagen type I. Cell monolayers were allowed to form under standard culture conditions over 7-8 days, with medium being refreshed every 2-3 days. The formation of cell monolayers was monitored by measuring the transepithelial electrical resistance (TEER) with an EVOM epithelial voltohmmeter with "chopstick" electrodes (World Precision Instruments, Sarasota, FL, USA).

Film samples with a surface area of 0.16 cm^2 (corresponding to 300 µg of TFV) were gently immersed in 0.5 mL of HBSS placed in the apical side of the cell monolayers. The basolateral side was pre-filled with 1.5 mL of HBSS. Inserts were maintained at 37 °C under orbital shaking (100 rpm) and the concentration of TFV in the basolateral side was monitored over time and up to 4 h by periodically collecting 0.5 mL samples and replacing it with fresh HBSS. At the end of the experiment, the inserts were collected, rinsed with HBSS and extracted with 1 mL of dimethyl sulphoxide in order to extract TFV associated with the cell monolayers [31, 32]. The permeability and cell monolayer retention of free TFV (300 µg) diluted in HBSS were also determined for comparison purposes. The amount of TFV in all samples was quantified by UV spectrophotometry at 260 nm. The apparent permeability coefficient (P_{app}) was calculated from permeability profiles according to:

$$P_{app} = \frac{Q}{A \cdot C \cdot t} \quad \text{(Eq. 2)}$$

where *Q* is the final amount of TFV permeated (μg), *A* is the area of the membrane (cm^2), *C* is the initial concentration of drug in the apical side (μg/mL) and *t* is the experiment time (s) [32]. All experiments were performed in triplicate.

2.5. Statistical analysis

One-way ANOVA with Tukey's post-hoc test was used to compare values of mucoadhesion time. Mechanical properties calculated from texture analysis data were compared by two-way ANOVA with Tukey's post-hoc test. Permeability profiles were compared by one-way ANOVA with Tukey's post-hoc test. Two-way ANOVA with Dunnett's post-hoc test was used to compare values of P_{app} and drug associated to cell monolayers. Analysis was performed with Prism® v. 5.03 (GraphPad Software, La Jolla, CA, USA). Values of $p < 0.05$ were considered as denoting significance. All results are presented as mean ± standard deviation (SD), unless otherwise mentioned.

3. RESULTS AND DISCUSSION

3.1. Technological characterisation

3.1.1. Drug release

We have shown recently that HPMC and zein films could be useful for producing vaginal films featuring prolonged release of TFV for as much as 120 h [19]. Contrary to what is usually sought in the field of microbicides, i.e. films presenting almost complete drug release within a few minutes after being placed in aqueous media, HPMC and zein films could provide an interesting platform for developing coitus-independent microbicides. Still, enhancing drug release when the HIV is deposited in the vagina by semen could further contribute to increasing the success of transmission blockage by such films. One particular way of achieving this goal encompasses the use of different plasticisers and their blends in order to confer pH-dependent drug release properties to HPMC and zein films. Indeed, our data highlighted the notable differences in TFV release according to selected plasticiser: films containing PEG sustained drug release in SVF for 144 h, while lactic acid reduced this time to only 12 h (Figure 1) [19]. Glycerol and oleic acid showed intermediate behaviours. These performances could be explained by the nature of the plasticisers. Oleic acid is an amphiphilic molecule that may interact with zein via carboxylic acid group bonding to the terminal glutamine residues of the protein [33], thus providing a sandwich-like structure that makes films less permeable [34]. Lactic acid and glycerol are polar molecules which undergo solvation in aqueous media, irrespective of pH, and facilitate drug diffusion. PEG is also a polar molecule, but features roughly 4-times higher molecular weight. This may hinder penetration into the polymer/protein network and enable intermolecular interactions among HPMC and zein in such a manner that the medium presents less diffusivity within the film matrix [35]. Moreover, PEG has been reported as forming chemical bonds with zein, via protein PEGylation [36]. It was further observed that films containing acidic plasticisers (lactic acid or oleic acid) feature faster drug release in the SVF/SSF medium (Figure 1B). A particularly marked increase in TFV release at higher pH was evident for films containing oleic acid, resulting in the only f_2 value lower than 50 for drug release comparisons of films containing a single plasticiser (Table 2). This suggests that oleic acid can be a good initial candidate plasticiser for providing pH-sensitive behaviour, while its combination with PEG can be relevant for reducing drug release at acidic pH. The comparison of f_2 values between films containing different single plasticisers further pointed out to differences in TFV release, except for the case of films with glycerol or oleic acid in SVF (*Supplementary Information, Table S1*).

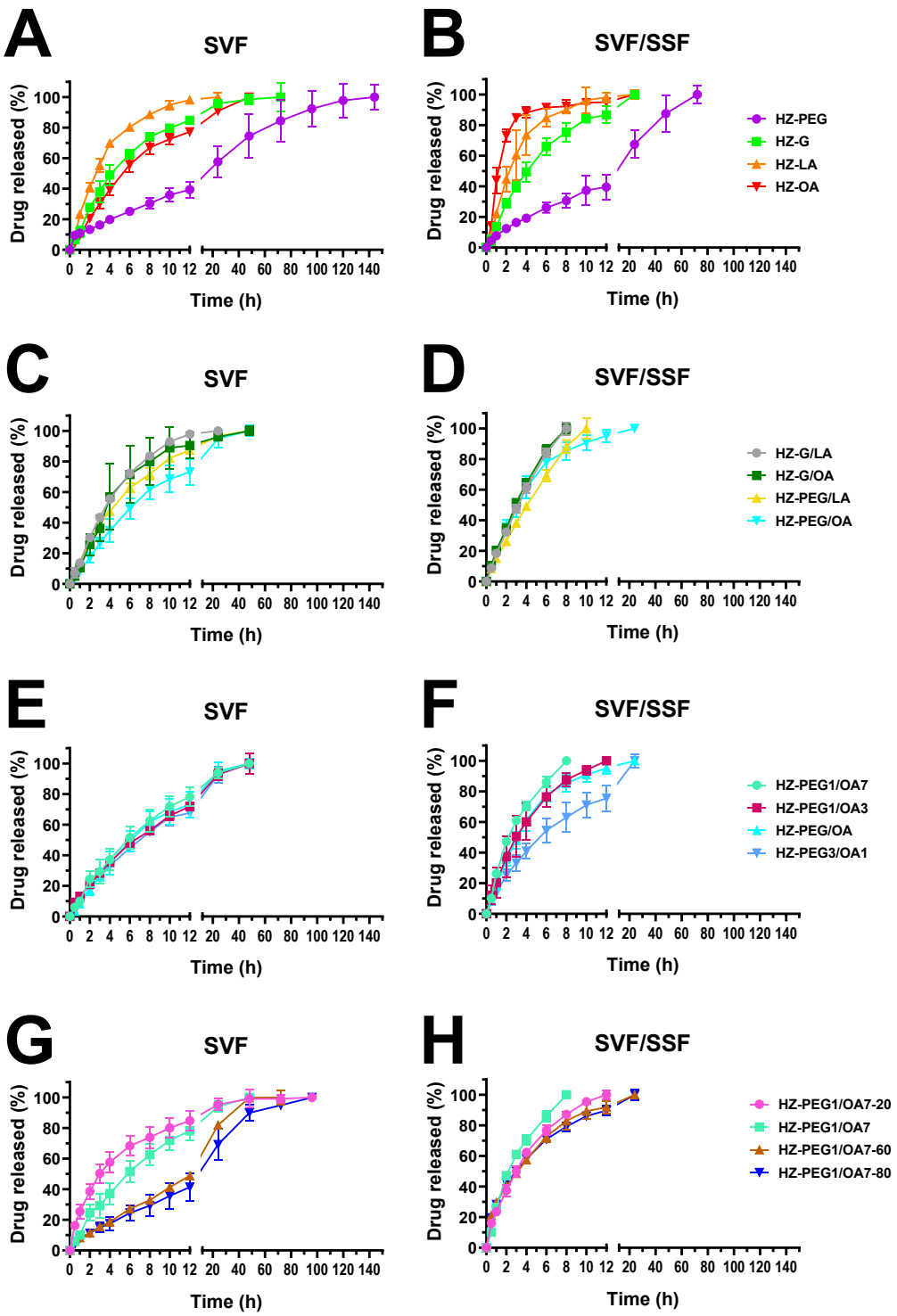

Figure 1. Drug release profiles of films prepared with a single plasticiser in SVF (A) and SVF/SSF (B); a combination of plasticisers in SVF (C) and SVF/SSF (D); a combination of PEG and oleic acid at different ratios in SVF (E) and SVF/SSF (F); and a combination of PEG and oleic acid with different ratios of matrix-forming materials/plasticisers in SVF (G) and SVF/SSF (H). Values for films prepared with PEG or glycerol as single plasticisers in SVF are adapted from previous published studies [19]. Results are presented as mean ± SD (*n*=3).

The study was proceeded by testing binary mixtures of plasticisers, namely one neutral and one acidic. Drug release profiles in SVF denoted intermediate behaviour between films including single plasticisers (Figure 1C). Films including PEG still trended towards slower drug release, especially when combined with oleic acid. Films prepared with binary mixtures of plasticisers also showed overall faster drug release in SVF/SSF than in SVF (Figure 1D). The comparison of f_2 values only pointed out to notable differences for films containing PEG and oleic acid, thus indicating the potential of such binary mixture to confer pH-dependent drug release behaviour (Table 2). Finally, comparison of f_2 values for different films revealed differences only for the mixtures of PEG/oleic acid in SVF (*Supplementary Information, Table S2*).

Table 2. Values of similarity factor (f_2) calculated from drug release profiles in SVF and in SVF/SSF. Values of f_2 denoting differences are presented in bold.

Film	f_2
HZ-PEG	64.5
HZ-G	81.1
HZ-LA	73.9
HZ-OA	**20.1**
HZ-G/LA	55.3
HZ-G/OA	**49.8**
HZ-PEG/LA	59.8
HZ-PEG/OA	**35.7**
HZ-PEG1/OA7	**30.8**
HZ-PEG1/OA3	**34.8**
HZ-PEG3/OA1	58.1
HZ-PEG1/OA7-20	61.3
HZ-PEG1/OA7-60	**22.8**
HZ-PEG1/OA7-80	**22.2**

The combination PEG/oleic acid was further tested at additional ratios (1:7, 1:3 and 3:1) for preparing films. There were no significant differences among these films regarding TFV release in SVF (Figure 1E). We hypothesized that, during the formation of films, oleic acid first interacts with zein and only later on with PEG. Thus, the structure formed by oleic acid and zein dominates, as can be seen when comparing SEM micrographs of films prepared with oleic acid alone or combining PEG and oleic acid (*Supplementary Information, Figures S1 and S2*). Values of comparison by f_2 (*Supplementary Information, Table S3*), indicate negligible differences among films. When considering data for SVF/SSF, increasing amounts of oleic acid led to faster release of TFV (Figure 1F and Table 2). Only films containing PEG/oleic acid in a ratio of 3:1 showed to be significantly different from the others; greater amounts of PEG appeared to have impaired TFV release in this medium (*Supplementary Information, Table S3*). Again, this supports the ability of oleic acid to yield pH-dependent drug release.

Overall, the higher differences among media in drug release of films with PEG/oleic acid at a ratio of 1:7 indicates that this formulation may be suitable for biphasic drug release. There were further evaluated different matrix-forming materials/plasticiser ratios in a last attempt to optimise the film formulation. Increasing amounts of plasticisers led to slower release of TFV in SVF (Figure 1G). The lowest tested concentration of plasticisers resulted in lower drug retention in SVF, which suggests poor plasticisation of HPMC and/or zein and, consequently, the control

of drug release is provided mostly by these non-pH-sensitive matrix-forming materials. Curiously, changes in drug release were minimal in the case of SVF/SSF (Figure 1H). Film with 80% of plasticisers (HZ-PEG1/OA7-80) presented the largest drug sustained release in SVF and was selected as the most suitable for pH-dependent release of TFV.

3.1.2. Swelling behaviour

We studied the ability of films to hydrate and erode in order to better understand drug release results. Although the swelling behaviour is mainly attributed to matrix-forming materials (typically polymers) [37, 38], our data showed notable differences between films featuring different plasticisers. Films containing lactic acid featured the greatest ability to take up both SVF and SVF/SSF (Figure 2A-B), thus confirming inferior plasticisation efficiency and justifying faster TFV release. Only films containing oleic acid showed substantial differences in swelling when comparing different media. In particular, these films were able to capture more fluid and maintain swelling for longer in SVF/SSF than in SVF. This suggests that changes to the sandwich structure of films plasticised with oleic acid are more pronounced with increasing pH, which could be implicated in the accelerated drug release observed in SVF/SSF.

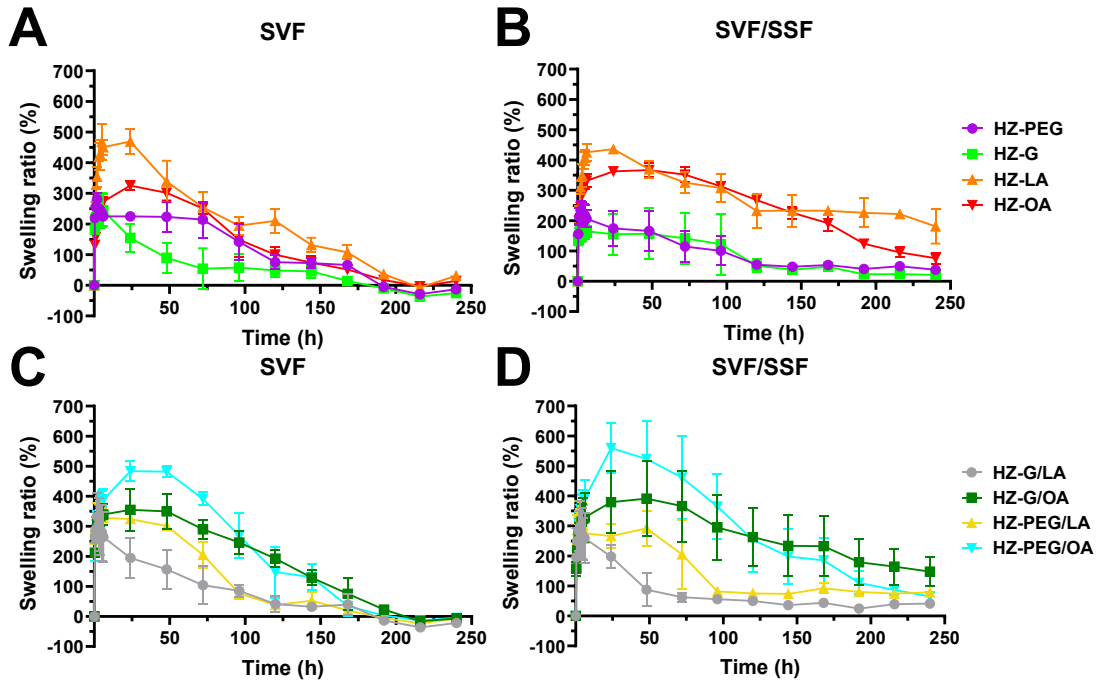

Figure 2. Swelling profiles of films prepared with a single plasticiser in SVF (A) and SVF/SSF (B), and films prepared with a combination of plasticisers in SVF (C) and SVF/SSF (D). Results are presented as mean ± SD (*n*=3).

Interestingly, the impact of acidic plasticisers was dissimilar in films featuring binary mixtures of plasticisers. Films with lactic acid presented the lowest ability to take up fluid (Figure 2C-D). This could be explained by the low interaction of lactic acid with matrix-forming materials and the consequent predominance of co-plasticisers (PEG or glycerol) in influencing swelling behaviour. Conversely, an increase in swelling was observed for films containing oleic acid combined with either PEG or glycerol. As previously described by Xu *et al*. [39], the interactions of oleic acid with zein during the fabrication process appear to be established at an earlier stage than those occurring between glycerol and the protein. The same effect may presumably be valid for films containing oleic acid and PEG. Thus, the layer-by-layer structure early established between zein

and oleic acid appears to be reinforced by the interaction of glycerol (or PEG) with hydrophilic domains of the matrix-forming protein, thus contributing to the enhancement of the swelling ability. Overall, films with oleic acid and a neutral plasticiser also featured more intense swelling in SVF/SSF than in SVF (Figure 2D).

3.1.3. Mucoadhesion

Drug dosage forms require to be retained in the vaginal cavity for a suitable amount of time after administration in order to effectively deliver active drug concentrations. Mucoadhesive properties of pharmaceutics are long regarded as beneficial in promoting vaginal residence, and have been mostly related with their polymeric content [40]. In the present work, it was set out to evaluate whether plasticisers could also affect the time of adhesion of films to the mucosa. Results for films prepared with a single plasticiser indicated that lactic acid and oleic acid feature the longest mucoadhesion time, remaining attached to mucosa during the full extent of the experiment (Figure 3). Mucoadhesion time was only 12.4 ± 0.6 days and 1.4 ± 0.8 days for films containing glycerol and PEG, respectively. Differences in swelling behaviour of these films may account for such observations [17, 41]. Moreover, acidic plasticisers may contribute to the overall negative charge at the surface of films and, thus, favour the establishment of electrostatic bonding with the mucosa [42]. The co-incorporation of acidic and neutral plasticisers appeared to feature an intermediate behaviour. Again, excessive swelling of films prepared with binary mixtures of plasticisers may partially hinder interactions between HPMC/zein and mucosa [43, 44]. Films optimised regarding drug release (80% of PEG/oleic acid at a ratio of 1:7) featured mucoadhesion time of 28 days, which further reinforce the relevance of oleic acid in enhancing adhesive interactions with mucosa.

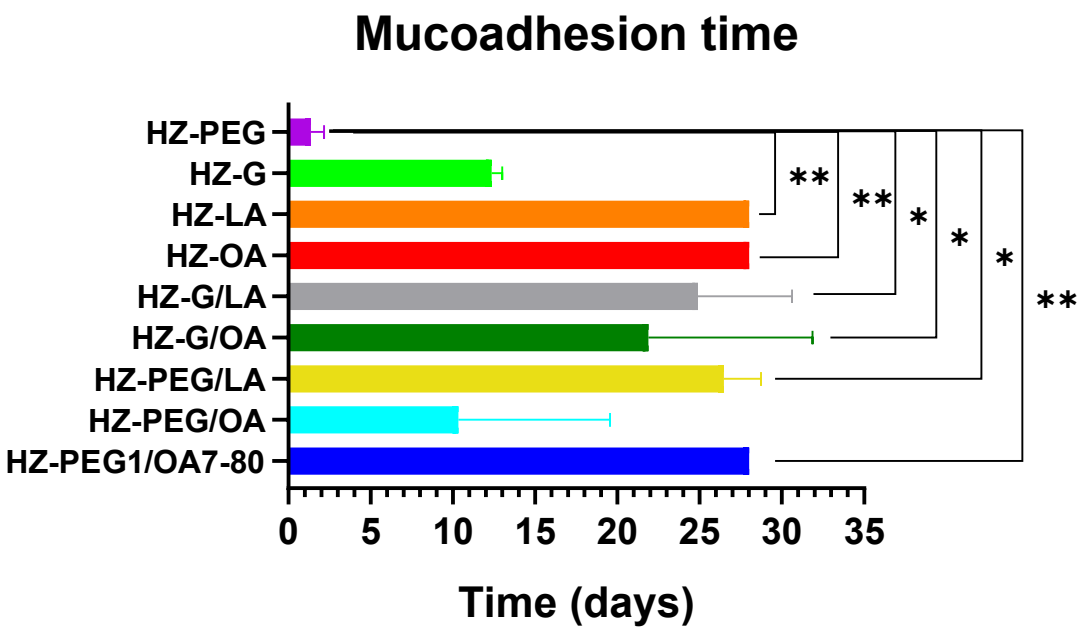

Figure 3. *Ex vivo* mucoadhesion time for films prepared with a single plasticiser, prepared with binary mixtures of plasticisers, and optimised regarding drug release (HZ-PEG1/OA7-80). Results are presented as mean ± SD (n=2). (*) and (**) denote $p < 0.05$ and $p < 0.01$, respectively.

3.1.4. Mechanical properties

All evaluated films showed suitable mechanical properties for their vaginal administration and use. Results obtained in the mechanical characterisation are discussed in *Supplementary Material* (*S2. Supplementary Results*).

3.2. Cellular studies

3.2.1. Cytotoxicity

Safety is a key aspect of microbicide development, and early *in vitro* assessment of formulation prototypes provides valuable hints on possible toxicity issues [45]. Complete cell viability profiles are presented in *Supplementary Information* (*Figure S4*). TFV and excipients included in optimised films were initially screened (Table 4 and *Supplementary Information, Figure S4*). In general, results confirm the relatively low toxicity potential of all ingredients, including TFV [46-48]. Marked decrease in cell viability was mostly observed only at the highest tested concentrations (10 mg/mL except for HPMC) and was correlated to changes in the properties of cell culture media. For example, we previously observed that PEG affects cell viability by markedly increasing osmolarity [47]. Oleic acid showed the highest decrease in cell viability, which could be attributable to the marked decrease in pH of media. However, this should not be a problem for human use due to the naturally acidic pH of the vaginal fluid.

Table 3. CC_{50} values of raw materials included in optimised films as determined using HeLa, Ca Ski and HEC-1-A cell lines.

Raw material	CC_{50} (mg/mL)		
	HeLa	Ca Ski	HEC-1-A
TFV	6.7	2.7	2.5
HPMC	> 1	> 1	> 1
Zein	6.7	7.8	> 10
Oleic acid	1.2	0.2	1.6
PEG	> 10	4.4	3.5

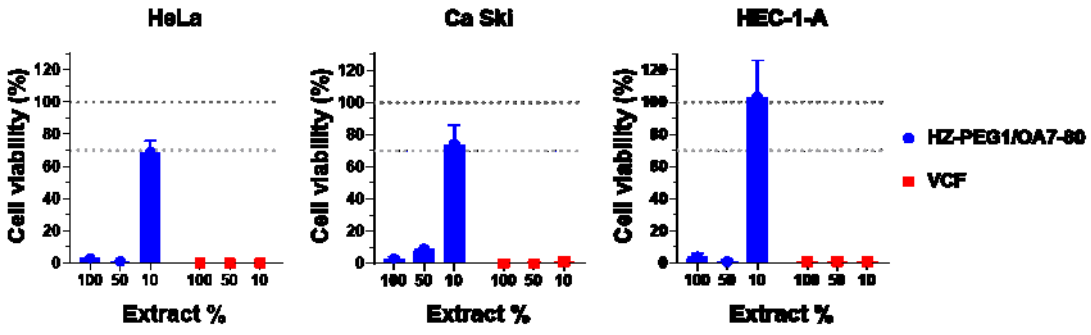

Figure 4. Viability of HeLa, Ca Ski and HEC-1-A cells upon exposure to extracts of optimised films and VCF®. Results are presented as mean ± SD (*n*=3).

More important, we assessed the cytotoxicity potential of optimised films by testing their extracts [49]. Data are presented in Figure 4. Extracts presented considerable cytotoxicity except for the 10% dilution (cell viability of approximately 70% or higher). The high content of oleic acid

in optimised films could account for these results. Again, changes in the pH of media may have influenced cell viability while in culture and, thus, additional toxicological assessment, including *in vivo*, is recommended for optimised films [46]. We also noted that optimised films were safer than the commercially-available VCF® Vaginal Contraceptive Film (Apothecus, Oyster Bay, NY, USA).

3.2.2. Permeability and epithelial cell retention

Different films, including the formulation optimised for pH-dependent drug release, were tested for their ability to influence the permeation of tenofovir across two relevant genital cell monolayer models. Experiments provide a proxy for systemic exposure (amount of permeated drug) and accumulation at the mucosa (amount of drug associated with the cell monolayer) [31, 32]. It should be noticed that the conditions used in these experiments mimic those observed upon ejaculation (pH of HBSS is 7.0-7.4). In general, transport of TFV across both cell monolayers was similar or lower for films as compared to the free compound (Figure 5A-B). TFV incorporated into films is not immediately available for crossing the cell monolayer, and this alone could account for observed permeability profiles. However, no correlation was apparent for drug release data and permeability when comparing different films, thus implying that transport across cell monolayers comprises complex phenomena and even be influenced by interactions between films or its ingredients and cells. Transport of TFV was generally faster across the Ca Ski model and in agreement with our previous observations [31, 32]. Calculated P_{app} values (Figure 5C) were in the range of 6.1 x 10^{-6} to 9.5 x 10^{-6} cm/s and 3.1 x 10^{-6} to 6.1 x 10^{-6} cm/s for Ca Ski and HEC-1-A cell monolayer models, respectively, and indicate that TFV is only mildly permeable [50]. We are unaware of permeability data reported for TFV using these two models. Still, our results were consistent with those reported for the permeability of TFV across Caco-2 colorectal cell monolayers when in solutions (P_{app} = 0.1 to 0.4 × 10^{-6} cm/s) [51-53], or ectocervical tissue when incorporated in vaginal gels (P_{app} = 2 to 3 × 10^{-6} cm/s) [54].

Microbicide drugs are supposed to exert their activity at the mucosal level. Therefore, assessing the ability of films to provide suitable high levels of TFV locally is relevant. Overall, tested films provided at least similar drug levels associated to cell monolayers in both Ca Ski and HEC-1-A models, as compared to the free drug (Figure 5D). In the case of the optimised film, no differences were observed. These data seem to assure that drug release at conditions mimicking the pH observed upon intravaginal ejaculation is fast enough to achieve suitable TFV levels for protection. Noticeably, films prepared with glycerol as single plasticiser led to a significantly higher amount of TFV associated with both type of cell monolayers, as compared with free TFV. Although requiring further investigation, we hypothesize that glycerol may interact directly with cells and facilitate intracellular accumulation of TFV. For instances, glycerol is known to inhibit multidrug resistance-associated proteins (MRPs) that could be involved in TFV efflux [55]. This film could suppose an alternative as vaginal microbicide. Although it does not provide pH-dependent release of TFV, it could increase protection against viral infection through the higher drug levels associated to cells. In addition, glycerol has also proved to be not toxic to female genital cells, such as HEC-1-A ones [19]. Therefore, this formulation also may be considered for future *in vivo* trials.

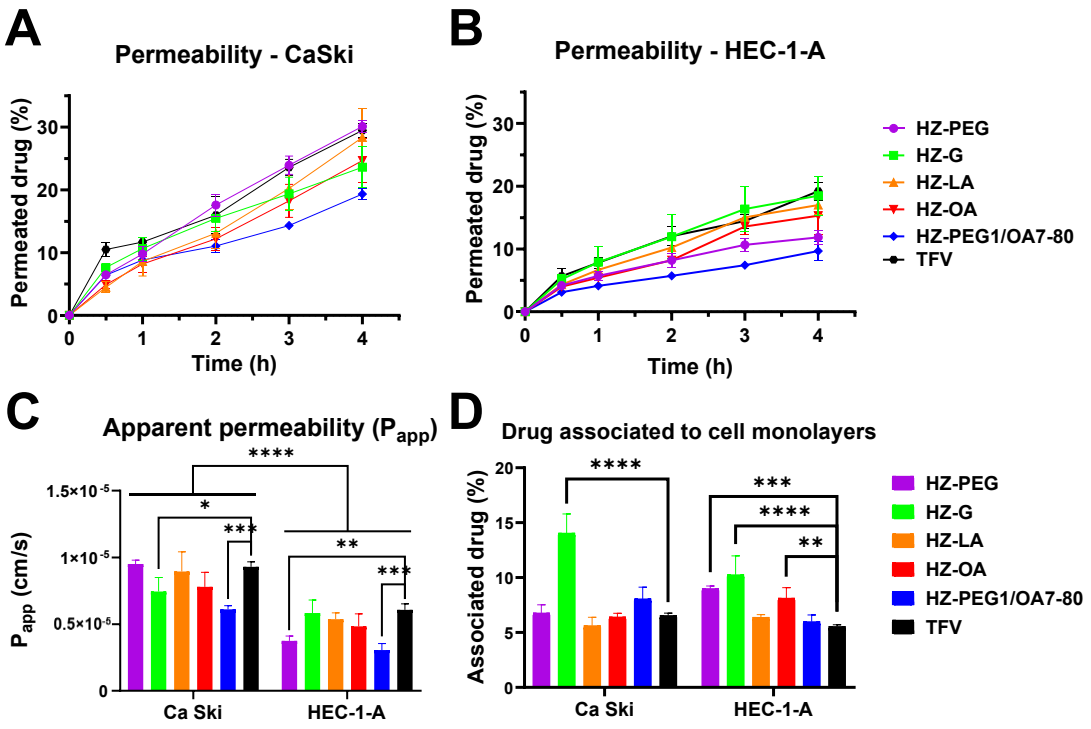

Figure 5. Permeability profiles of TFV across Ca Ski (A) and HEC-1-A (B) cell monolayers, and apparent permeability (C) and drug associated to cell monolayers (D), as mediated by different films or the free drug. Results are presented as mean ± SD (*n*=3). (*), (**), (***) and (****) denote $p < 0.05$, $p < 0.01$, $p < 0.001$ and $p < 0.0001$, respectively.

4. CONCLUSIONS

Films presenting pH-dependent drug release hold the potential to provide interesting microbicide products. The addition of different plasticisers to films prepared with the same matrix-forming ingredients (HPMC and zein) could modify the release profile of TFV. Films optimised with an 80% plasticising mixture of oleic acid and PEG in a ratio of 1:7 featured promising characteristics for developing semen-triggered microbicides. In these films, the presence of PEG helps holding drug release in SVF, while the content in oleic acid allows doubling TFV release rate when the medium is neutralised by seminal fluid. Oleic acid also provides to the formulation proper adhesion to vaginal mucosa and the presence of PEG gives mechanical properties suitable for comfortable intravaginal administration. Moreover, the formulation including these plasticisers allowed similar association levels of TFV to genital epithelial cell monolayers to those observed for the drug in solution, but possibly with lower systemic exposure.

On the other hand, film plasticised only with glycerol could be appropriate as a microbicide for immediate release of TFV. This film is able to double the amount of TFV associated to both Ca Ski and HEC-1-A cell monolayers, compared to free TFV. In consequence, its administration could increase the protection of women against sexual transmission of HIV. The nature of this plasticiser also provides acceptable mucoadhesive and mechanical properties to films, but it would require a more frequent administration dosage due to the not pH-dependent release of TFV.

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AUTHOR CONTRIBUTIONS

Fernando Notario-Pérez: Conceptualization, Investigation, Writing – Original Draft. Raúl Cazorla-Luna: Investigation. Araceli Martín-Illana: Investigation. Joana Galante: Investigation. Roberto Ruiz-Caro: Conceptualization, Writing – Review & Editing, Supervision. Bruno Sarmento: Conceptualization, Writing – Review & Editing, Supervision, Funding acquisition. José das Neves: Conceptualization, Writing – Review & Editing, Supervision. María-Dolores Veiga: Conceptualization, Writing – Review & Editing, Supervision, Project administration, Funding acquisition. All the authors have approved the final article.

DECLARATIONS OF INTEREST

Declarations of interest: none

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Supplementary Information

Influence of plasticisers on pH-dependent drug release and cellular interactions of hydroxypropyl methylcellulose/zein vaginal anti-HIV films containing tenofovir

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S1. Supplementary methods

S1.1. Mechanical properties

A TA.TXT*plus* Texture Analyser (Stable Micro Systems, Surrey, UK) on compression mode, with a 30 kg load cell, was used to evaluate the mechanical properties of films. Burst strength and distance at burst were determined using a cylindrical probe (5 mm circular diameter) moving at 0.5 mm/s for puncturing and rupturing a film sample fixed in perpendicular position [1, 2]. Experiments were carried out in quadruplicate for each film. Tensile strength and elongation at break were also determined by using the equipment in tension mode with a 5 kg load cell. Film samples (2 x 4 cm) were placed between tensile grips initially separated by 2.5 cm and moved at 1 mm/s until the film collapsed. Experiments were performed in triplicate, and elongation per area and elastic modulus of the films were calculated.

S1.2. Scanning electron microscopy (SEM)

The surface of films was observed with a scanning electron microscope (JEOL JSM-6335F, JEOL Ltd., Tokyo, Japan) at an acceleration voltage of 15 kV. Films were coated with gold for 90 s in a high vacuum atmosphere. Micrographs were taken at 100x, 500x and 1000x magnifications. Intact and pre-wetted films were observed. In the last case, changes to the microstructure of films were assessed after immersion in SVF for 120 h followed by freeze-drying, as previously described by our group [3]. Freeze-dried samples were then analysed by SEM.

S2. Supplementary Results

S2.1. Drug release – f_2 comparison between different films

Table S1. Similarity factor (f_2) values for the comparison of release profiles obtained for films developed with a single plasticiser – polyethylene glycol (PEG), glycerol (G), lactic acid (LA) and oleic acid (OA) –, in either SVF or SVF/SSF. Values of f_2 lower than 50 are highlighted in bold.

Film comparisons		f_2
HZ-PEG – SVF	HZ-G – SVF	**25.7**
HZ-PEG – SVF	HZ-LA – SVF	**21.2**
HZ-PEG – SVF	HZ-OA – SVF	**30.2**
HZ-G – SVF	HZ-LA – SVF	**42.7**
HZ-G – SVF	HZ-OA – SVF	59.6
HZ-LA – SVF	HZ-OA – SVF	**34.8**
HZ-PEG – SVF/SSF	HZ-G – SVF/SSF	**25.3**
HZ-PEG – SVF/SSF	HZ-LA – SVF/SSF	**19.6**
HZ-PEG – SVF/SSF	HZ-OA – SVF/SSF	**15.4**
HZ-G – SVF/SSF	HZ-LA – SVF/SSF	**40.4**
HZ-G – SVF/SSF	HZ-OA – SVF/SSF	**24.1**
HZ-LA – SVF/SSF	HZ-OA – SVF/SSF	**36.2**

Table S2. Similarity factor (f_2) values for the comparison of release profiles obtained for films developed with a blend of plasticisers – polyethylene glycol (PEG), glycerol (G), lactic acid (LA) and oleic acid (OA) –, in either SVF or SVF/SSF. Values of f_2 lower than 50 are highlighted in bold.

Film comparisons		f_2
HZ-G/LA – SVF	HZ-G/OA – SVF	71.0
HZ-G/LA – SVF	HZ-PEG/LA – SVF	57.7
HZ-G/LA – SVF	HZ-PEG/OA – SVF	**38.8**
HZ-G/OA – SVF	HZ-PEG/LA – SVF	61.4
HZ-G/OA – SVF	HZ-PEG/OA – SVF	**41.9**
HZ-PEG/LA – SVF	HZ-PEG/OA – SVF	**48.5**
HZ-G/LA – SVF/SSF	HZ-G/OA – SVF/SSF	79.1
HZ-G/LA – SVF/SSF	HZ-PEG/LA – SVF/SSF	52.3
HZ-G/LA – SVF/SSF	HZ-PEG/OA – SVF/SSF	61.6
HZ-G/OA – SVF/SSF	HZ-PEG/LA – SVF/SSF	**48.6**
HZ-G/OA – SVF/SSF	HZ-PEG/OA – SVF/SSF	70.2
HZ-PEG/LA – SVF/SSF	HZ-PEG/OA – SVF/SSF	56.5

Table S3. Similarity factor (f_2) values for the comparison of release profiles obtained for films combining polyethylene glycol (PEG) and oleic acid (OA) at different ratios, in either SVF or SVF/SSF. Values of f_2 lower than 50 are highlighted in bold.

Reference	Problem	f_2
HZ-PEG1/OA7 – SVF	HZ-PEG1/OA3 – SVF	69.6
HZ-PEG1/OA7 – SVF	HZ-PEG/OA – SVF	73.0
HZ-PEG1/OA7 – SVF	HZ-PEG3/OA1 – SVF	62.6
HZ-PEG1/OA3 – SVF	HZ-PEG/OA – SVF	72.8
HZ-PEG1/OA3 – SVF	HZ-PEG3/OA1 – SVF	76.4
HZ-PEG/OA – SVF	HZ-PEG3/OA1 – SVF	73.1
HZ-PEG1/OA7 – SVF/SSF	HZ-PEG1/OA3 – SVF/SSF	54.8
HZ-PEG1/OA7 – SVF/SSF	HZ-PEG/OA – SVF/SSF	53.1
HZ-PEG1/OA7 – SVF/SSF	HZ-PEG3/OA1 – SVF/SSF	**33.3**
HZ-PEG1/OA3 – SVF/SSF	HZ-PEG/OA – SVF/SSF	81.8
HZ-PEG1/OA3 – SVF/SSF	HZ-PEG3/OA1 – SVF/SSF	**40.3**
HZ-PEG/OA – SVF/SSF	HZ-PEG3/OA1 – SVF/SSF	**48.2**

Table S4. Similarity factor (f_2) values for the comparison of release profiles obtained for films plasticised with polyethylene glycol (PEG) and oleic acid (OA) at ratio 1:7, manufactured with different ratios polymer/plasticiser, in either SVF or SVF/SSF. Values of f_2 lower than 50 are highlighted in bold.

Reference	Problem	f_2
HZ-PEG1/OA7-20 – SVF	HZ-PEG1/OA7 – SVF	**43.9**
HZ-PEG1/OA7-20 – SVF	HZ-PEG1/OA7-60 – SVF	**25.8**
HZ-PEG1/OA7-20 – SVF	HZ-PEG1/OA7-80 – SVF	**23.6**
HZ-PEG1/OA7 – SVF	HZ-PEG1/OA7-60 – SVF	**35.6**
HZ-PEG1/OA7 – SVF	HZ-PEG1/OA7-80 – SVF	**31.7**
HZ-PEG1/OA7-60 – SVF	HZ-PEG1/OA7-80 – SVF	62.3
HZ-PEG1/OA7-20 – SVF/SSF	HZ-PEG1/OA7 – SVF/SSF	55.6
HZ-PEG1/OA7-20 – SVF/SSF	HZ-PEG1/OA7-60 – SVF/SSF	69.0
HZ-PEG1/OA7-20 – SVF/SSF	HZ-PEG1/OA7-80 – SVF/SSF	66.2
HZ-PEG1/OA7 – SVF/SSF	HZ-PEG1/OA7-60 – SVF/SSF	**37.6**
HZ-PEG1/OA7 – SVF/SSF	HZ-PEG1/OA7-80 – SVF/SSF	**38.7**
HZ-PEG1/OA7-60 – SVF/SSF	HZ-PEG1/OA7-80 – SVF/SSF	82.1

S2.2. SEM micrographs

The surface morphology of films was observed by SEM. Differences among films with a single plasticiser were generally mild (Figure S1). Micropores can be traced in films prepared with glycerol or lactic acid at the solid-state. The presence of micropores may be implicated in facilitated release of TFV from these films. Conversely, films plasticised with PEG or oleic acid featured completely smooth surface, which agrees with the structure observed for zein films by others [4].

After swelling and freeze-drying the surface was eroded and heterogeneous. Films with lactic acid appeared to be more porous, thus supporting the onset of facilitated water diffusion. Less and smaller pores were apparent in films with PEG and glycerol, which – as seen in swelling tests

(Figure 2 in main text) – indicate lower water influx. Finally, films with oleic acid stood out as the most different ones after swelling. Large gaps could be observed, but no micropores, which suggests the unique disposition of zein when plasticised with oleic acid [5]. The presence of hydrophobic groups oriented towards outer layers may limit the formation of micropores when in contact with SVF.

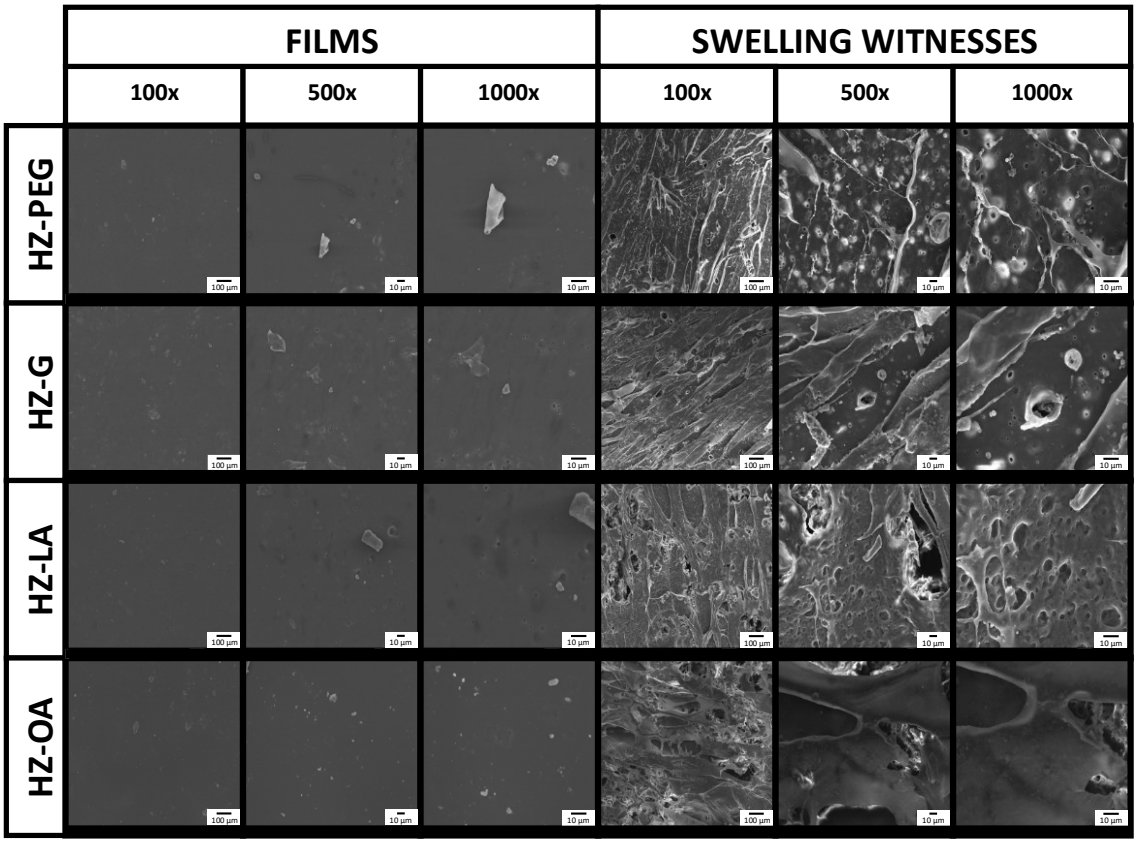

Figure S1. Scanning electron microscopy photographs of films prepared with a single plasticiser – polyethylene glycol (PEG), glycerol (G), lactic acid (LA) and oleic acid (OA) – at the solid-state (left), and after 120 h immersion in SVF and freeze-drying (right). Representative micrographs were taken with an acceleration voltage of 15 kV and are presented at magnifications of 100x, 500x and 1000x.

Films prepared with a binary mixture of plasticisers featured smooth surface, without the presence of notable micropores (Figure S2). The combination of plasticisers appears to originate a more compact and homogeneous structure, namely when comparing with films prepared with either glycerol or lactic acid. Differences were more apparent after immersion in SVF. Films with glycerol and lactic acid were more porous (pores around 10 µm or smaller), which could help explaining faster drug release. Films with glycerol/oleic acid and PEG/lactic acid also featured small micropores, although less numerous, as well as aggregates of spherical particles. As observed by other authors, These are in agreement with previous observations of the formation of zein aggregates due to the polymerisation of protein chains [6]. Finally, films with PEG and oleic acid showed large morphological differences, namely heterogeneous surface and big surface gaps. Curiously, these features are similar to those of film prepared only with oleic acid, thus suggesting that this plasticiser is key in defining the structure of films containing PEG and oleic acid.

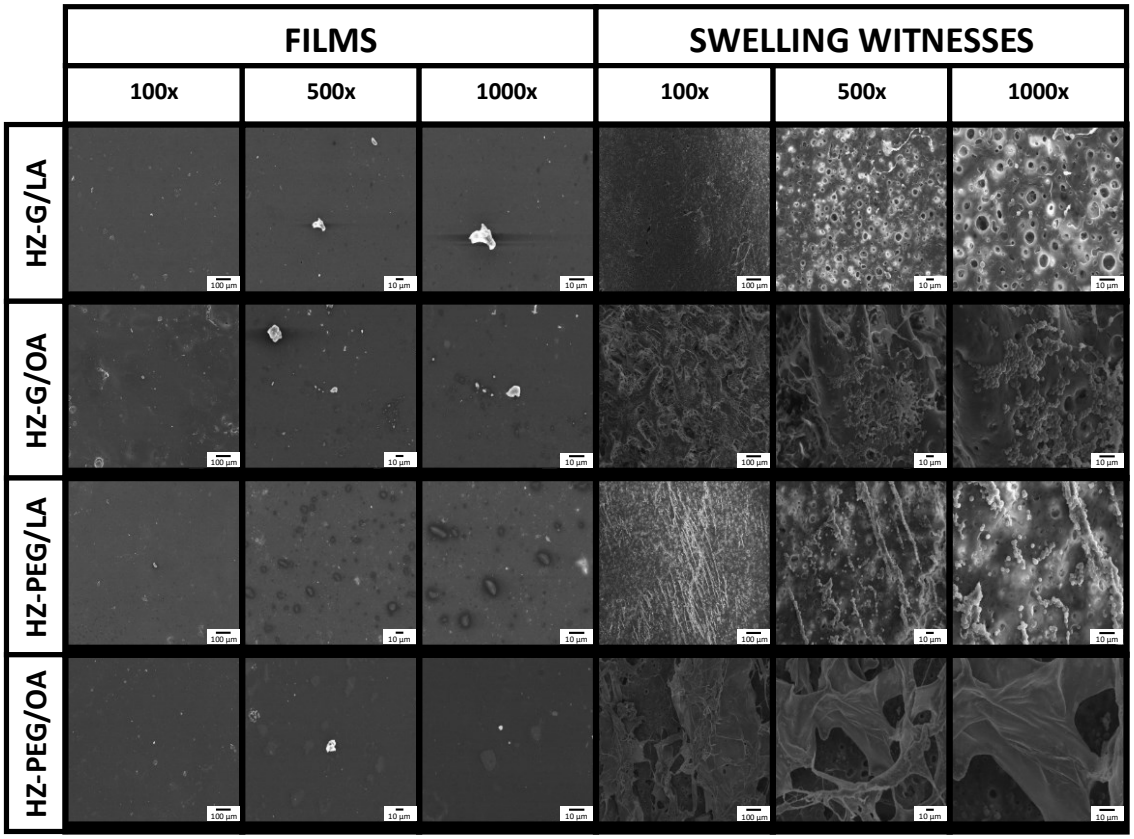

Figure S2. Scanning electron microscopy photographs of films combining two plasticisers – polyethylene glycol (PEG), glycerol (G), lactic acid (LA) or oleic acid (OA) – at the solid-state (left), and after 120 h immersion in SVF and freeze-drying (right). Representative micrographs were taken with an acceleration voltage of 15 kV and are presented at magnifications of 100x, 500x and 1000x.

Optimised films containing 80% of a binary mixture of plasticisers (PEG/oleic acid at a ratio of 1:7) had a homogenous and smooth surface, with almost no pores being observed before swelling (Figure S3). This agrees with the structure obtained for films combining equal amounts of PEG and oleic acid. The presence of homogeneously distributed pores (10 µm or smaller) was noted after immersion in SVF. These smaller pores (as compared to films prepared with equal amounts of plasticisers) may account for the slower release of TFV from optimised films.

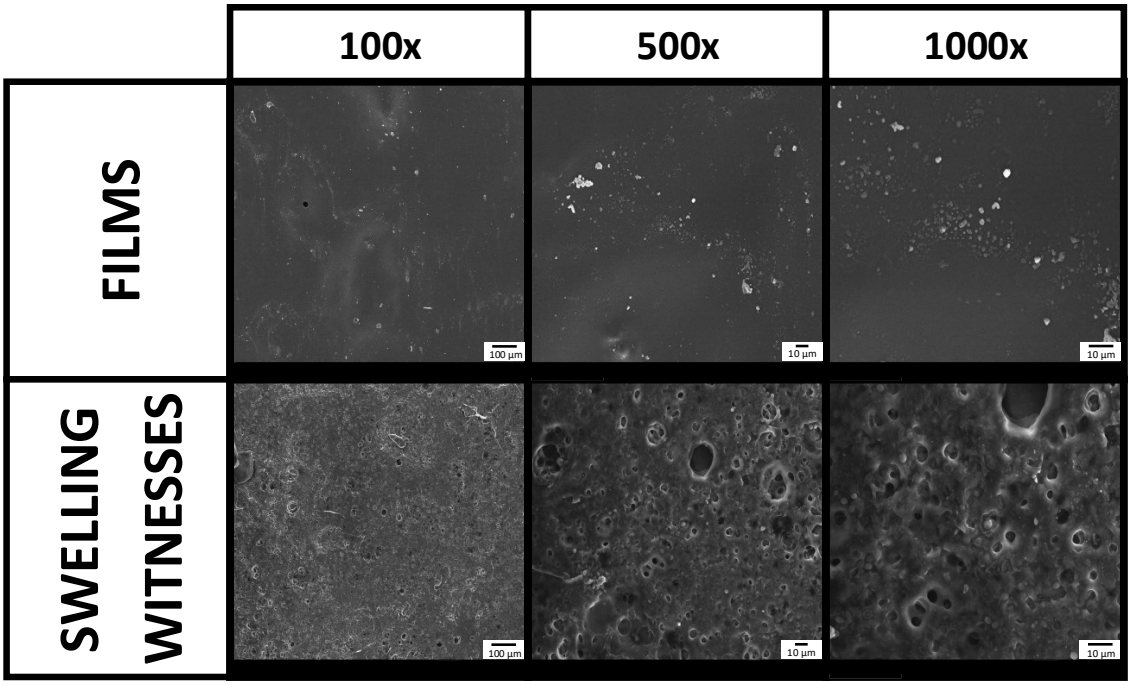

Figure S3. Scanning electron microscopy photographs of films combining 80% plasticisers (PEG and oleic acid at a ratio of 1:7) at the solid-state (top), and after 120 h immersion in SVF and freeze-drying (bottom). Representative micrographs were taken with an acceleration voltage of 15 kV and are presented at magnifications of 100x, 500x and 1000x.

S2.3. Mechanical properties

Films plasticised with lactic acid or PEG featured overall improved mechanical properties as compared to those incorporating either oleic acid or glycerol (Table 3). In particular, these last presented poorer values of resistance and elasticity. Again, the use of binary mixtures of plasticisers appeared to be helpful in providing acceptable mechanical properties to films, and even confirming previous reports of the synergistic interactions of these plasticisers in films containing zein [7]. The formulation optimised regarding drug release presented good mechanical properties, indicating proper plasticisation. The ratio distance/force and the elongation were greater for optimised films and may be contribute to their facile administration and comfortable use [1]. Statistical analysis proved significant differences among films' mechanical properties. Force at burst, and particularly elongation at break, were the parameters most affected as a function of the plasticiser, as shown in multiple comparisons Tukey's test (*Table S6*). The other parameters did not show differences among compared films.

Table S5. Mechanical properties of films prepared with a single plasticiser, prepared with a binary mixture of plasticisers, and optimised regarding drug release (HZ-PEG1/OA7-80), as evaluated using puncture and stretching tests. Results are presented as mean ± SD (*n*=3).

Film	Puncture test		Stretching test			
	Force at burst (N)	Distance at burst (mm)	Tensile strength (N/cm^2)	Elongation at break (%)	Elongation per area ($\%/cm^2$)	Elastic modulus (N/cm^2)
HZ-PEG	16.03 ± 8.07	2.30 ± 0.08	3.38 ± 0.13	57.1 ± 5.4	7.1 ± 0.7	1.77 ± 0.37
HZ-G	6.37 ± 2.51	1.18 ± 0.10	4.30 ± 0.33	59.2 ± 26.1	7.4 ± 3.3	0.30 ± 0.15
HZ-LA	29.60 ± 9.38	3.54 ± 0.99	1.43 ± 0.26	136.5 ± 11.4	17.1 ± 1.4	0.96 ± 0.17
HZ-OA	5.81 ± 0.89	1.65 ± 0.13	6.61 ± 1.62	48.3 ± 28.5	6.0 ± 3.6	0.73 ± 0.35
HZ-G/LA	29.08 ± 2.45	3.41 ± 0.23	2.58 ± 0.09	144.5 ± 19.7	18.1 ± 2.5	1.27 ± 0.41
HZ-G/OA	8.65 ± 1.28	1.26 ± 0.08	5.66 ± 0.15	80.6 ± 0.3	10.1 ± 0.0	0.38 ± 0.08
HZ-PEG/LA	10.42 ± 2.17	2.25 ± 0.24	2.61 ± 0.43	114.5 ± 14.0	14.3 ± 1.7	1.41 ± 0.22
HZ-PEG/OA	10.37 ± 1.42	1.45 ± 0.26	8.35 ± 0.05	6.7 ± 1.3	0.8 ± 0.2	4.16 ± 0.39
HZ-PEG1/OA7-80	4.41 ± 0.31	1.35 ± 0.16	4.25 ± 0.12	19.1 ± 3.9	2.4 ± 0.5	0.66 ± 0.21

Table S6. p-values obtained in Tukey's multiple comparisons test. Values of $p < 0.05$ (highlighted in bold) were considered as denoting significance.

Film Comparison	p-value	
	Force at burst	Elongation at break
HZ-PEG vs. HZ-G	0.4484	>0.9999
HZ-PEG vs. HZ-LA	0.0739	**<0.0001**
HZ-PEG vs. HZ-OA	0.3691	0.7505
HZ-G vs. HZ-LA	**<0.0001**	**<0.0001**
HZ-G vs. HZ-OA	>0.9999	0.4811
HZ-LA vs. HZ-OA	**<0.0001**	**<0.0001**
HZ-PEG vs. HZ-PEG/LA	0.9446	**<0.0001**
HZ-PEG vs. HZ-PEG/OA	0.9417	**<0.0001**
HZ-G vs. HZ-G/LA	**<0.0001**	**<0.0001**
HZ-G vs. HZ-G/OA	0.9999	**0.0022**
HZ-LA vs. HZ-G/LA	>0.9999	0.8354
HZ-LA vs. HZ-PEG/LA	**0.0013**	**0.0014**
HZ-OA vs. HZ-G/OA	0.9994	**<0.0001**
HZ-OA vs. HZ-PEG/OA	0.9841	**<0.0001**
HZ-G/LA vs. HZ-G/OA	**0.0004**	**<0.0001**
HZ-G/LA vs. HZ-PEG/LA	**0.0020**	**<0.0001**
HZ-PEG/LA vs. HZ-PEG/OA	>0.9999	**<0.0001**
HZ-G/OA vs. HZ-PEG/OA	>0.9999	**<0.0001**
HZ-PEG vs. HZ-PEG1/OA7-80	0.2059	**<0.0001**
HZ-OA vs. HZ-PEG1/OA7-80	>0.9999	**<0.0001**
HZ-PEG/OA vs. HZ-PEG1/OA7-80	0.9226	0.3020

S2.4. Cytotoxicity test

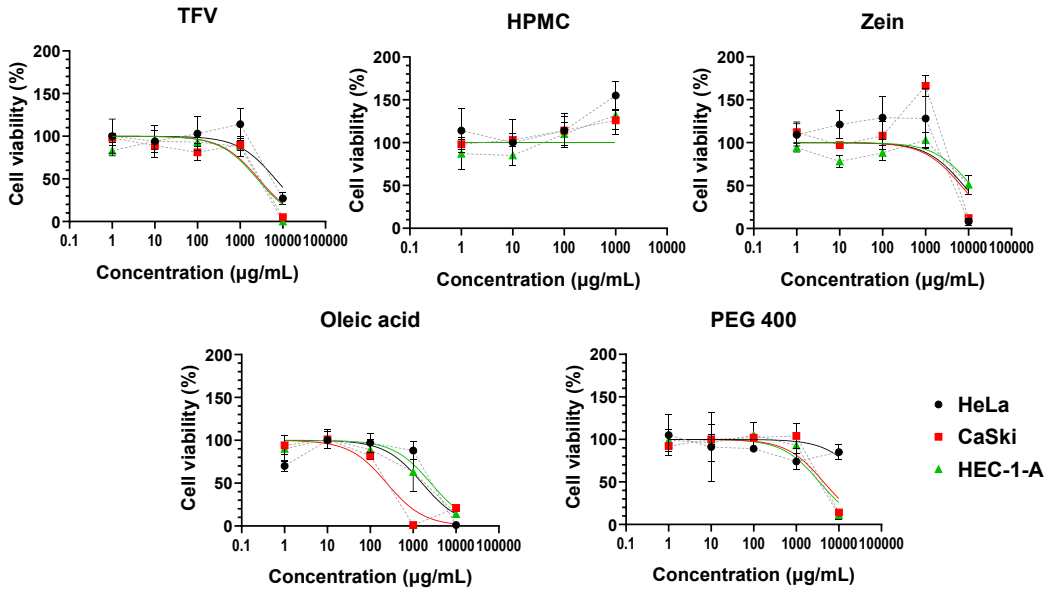

Figure S4. Viability of HeLa, Ca Ski and HEC-1-A cell lines upon exposure to TFV or different excipients, as assessed by the resazurin metabolism assay. Results are presented as mean ± standard deviation (*n*=3). Solid lines represent log-logistic regressions for each plotted data set.

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CAPÍTULO IX

DEVELOPMENT OF pH-SENSITIVE VAGINAL FILMS BASED ON METHACRYLATE COPOLYMERS FOR TOPICAL HIV-1 PRE-EXPOSURE PROPHYLAXIS

Development of pH-sensitive vaginal films based on methacrylate copolymers for topical HIV-1 pre-exposure prophylaxis

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ABSTRACT

The interest in "smart" vaginal microbicides as a strategy to protect women from sexual transmission of human immunodeficiency virus type 1 (HIV-1) is growing. The concept is based on the development of products that are able to feature low drug release in acidic media – such as the vaginal fluid –, but switch to a fast release profile when the medium becomes neutral or slightly alkaline. This mimics the rapid pH rise occurring in the vagina after sexual intercourse due to the presence of seminal fluid. Semen is the main vehicle for HIV-1, and increasing antiretroviral drug levels at the vagina upon ejaculation may contribute to enhanced protection against viral sexual transmission. In this work, it is explored the use of different pharmaceutical grade methacrylic acid-based polymers (Eudragit® RL PO, RS PO, L 100 and S 100) for developing vaginal films featuring pH-dependent release of the antiretroviral drug tenofovir (TFV). Eudragit® L 100 and Eudragit® S 100 containing triethyl citrate as plasticiser were shown suitable for manufacturing films with optimal dual *in vitro* drug release behaviour. TFV-release can be held for several days after film administration and all the drug is released in a few hours at conditions simulating ejaculation. Films also featured mechanical properties deemed suitable for comfortable vaginal administration and use. Two optimized films were further assessed using HEC-1-A and Ca Ski cell monolayer models and shown to possess favourable drug permeability profiles and drug levels associated to cell monolayer as compared to free TFV. Overall, developed pH-dependent films containing tenofovir may constitute promising candidate "smart" vaginal microbicides for protecting women from sexual HIV transmission.

1. INTRODUCTION

The development of vaginal microbicides to protect women from sexual transmission of human immunodeficiency virus (HIV) has been a widely explored research field over the last two decades. Several active compounds have been evaluated as potential microbicide agents including surfactants, polyanions, acidifiers and monoclonal antibodies [1]. Nevertheless, only highly potent antiretroviral drugs, such as tenofovir (TFV) or dapivirine, have been shown useful in decreasing male-to-female transmission of HIV through the cervicovaginal route [2-4]. TFV is a highly hydrophilic nucleotide reverse transcriptase inhibitor with potent antiretroviral activity, and a forefront microbicide candidate [1, 5]. Its vaginal use at 1% in different vaginal gels has been shown safe in human clinical trials [6, 7].

Apart from the intrinsic anti-HIV activity of microbicide compound candidates, their success is dependent on their suitable formulation as vaginal products [8]. Research has mainly focused on the development of several dosage forms loaded with antiretroviral drugs, namely vaginal gels, freeze-dried gels, tablets, rings or films [9-13]. One of the main problems of these formulations relates to the inability to sustain local drug levels following administration, with the exception of rings. In this last case, drug levels at the vagina are maintained for weeks while the ring is in place. Still, many women appear to dislike the need to use the ring in a continuous manner, and on-demand microbicide products (i.e., only used when perceived as necessary) may be preferred [14]. Thus, “smart” microbicides that can be used as on-demand products, but are also enabled by actual sexual intercourse could be an interesting approach for optimizing user’s adherence and protection. Seminal fluid is characterized by its alkaline nature (pH 7-8) and high buffering capacity, and is able to raise the pH of acidic vaginal fluids (pH 4-5) to neutral/slightly alkaline values [15]. Increased vaginal pH due to the deposition of semen upon ejaculation has been long identified as an event that could trigger changes in microbicide formulations leading to enhanced drug release. For example, nanoparticles based on acrylic derivatives have already been used to modify the release of TFV in a pH-dependent manner [16]. Freeze-dried bigels manufactured with pectin and sesame oil also exhibited the ability to accelerate the release of this drug in an alkaline medium [17].

Vaginal films have been relatively unexplored as pH-sensitive dosage forms for developing microbicides. They comprise a thin soft and flexible sheet that is typically formed by one of several matrix-forming polymers where drugs are dispersed. Films have been traditionally designed in order to feature fast-dissolving properties and quickly release incorporated drugs [18, 19]. There is, however, an increasing interest in films that are able to sustain drug release and provide a wider time-window of protection against HIV transmission [20, 21]. The use of matrix-forming polymers that feature different aqueous solubility according to pH has been described for drug delivery applications [22]. Polymers that are poorly soluble below pH≈6 but readily dissolve above this value are good candidates for developing semen-triggered microbicides. The commercially available Eudragit® excipient series (acrylic derivatives based on polymethacrylate copolymers that ate commonly used as coating agents for oral dosage forms) includes several interesting candidates for this last purpose [23].

In this paper, we describe the systematic evaluation of four of these worldwide approved excipients, namely Eudragit® RS PO (ammonium methacrylate copolymer type B), Eudragit® RL PO (ammonium methacrylate copolymer), Eudragit® S 100 (methacrylic acid/methyl methacrylate copolymer, ratio 1:2) and Eudragit® L 100 (methacrylic acid/methyl methacrylate copolymer, ratio 1:1) as potential matrix-forming polymers for vaginal films incorporating TFV. In particular, films should enable pH-dependent *in vitro* drug release: slower in acidic simulated

vaginal fluid (SVF) and faster in a slightly alkaline mixture of SVF with simulated seminal fluid (SSF). Optimized films were further assessed for their mechanical properties, cytotoxicity, and drug permeability and membrane retention using two *in vitro* cell monolayer models relevant to microbicide development.

2. MATERIALS AND METHODS

2.1. Materials

Eudragit® RS PO (ERS; lot: G120238035), Eudragit® RL PO (ERL; lot: G120536083), Eudragit® S 100 (ES; lot: B071005090) and Eudragit® L 100 (EL; lot: B110603006) were kindly supplied by Evonik (Essen, Germany). Glycerol (lot: 0000539368) and L-(+)-lactic acid (lot: 0001552193) were purchased from Panreac (Barcelona, Spain). Polyethylene glycol 400 (PEG; Kollisolv® PEG E 400; lot: BCBQ6662V), triethyl citrate (TEC; lot: BCBN8745V), tributyl citrate (TBC; lot: BCBP4709V), oleic acid (lot: BCBR1202V), rat tail collagen type I and resazurin were acquired from Sigma-Aldrich (St. Louis, MO, USA). TFV (lot: FT104801401) was supplied by Carbosynth Limited (Berkshire, UK). Dulbecco's Modified Eagle medium (DMEM) was purchased from Lonza (Verviers, Belgium), RPMI 1640 medium and Hank's balanced salt solution (HBSS) from Gibco (ThermoFisher Scientific, Waltham, MA, USA), and McCoy's 5A medium from Alfagene (Carcavelos, Portugal). Fetal bovine serum (FBS), penicillin and streptomycin were acquired from Invitrogen (ThermoFisher Scientific, Waltham, MA, USA). Acetone, ethanol, methanol, isopropanol and all other reagents used in this study were of analytical grade and used without further purification. Demineralised water was used in all cases.

2.2. Preparation of films

Films were prepared by solvent casting method [20]. Briefly, 250 mg of Eudragit® and 30 mg of TFV were individually weighed for each film and placed in individual round silicone templates (43 mm in diameter, corresponding to an area of 14.5 cm^2). The amount of plasticiser incorporated per film was 50, 100, 150 or 200 mg, corresponding to 20%, 40%, 60% and 80% (% w/w) of the total weight of polymer. The plasticiser was dissolved in 10 mL of isopropanol and added to each silicone template. All components were allowed to dissolve and the templates were left at room temperature until complete solvent evaporation. Films were then peeled off and used without further processing. Their composition is detailed in Table 1.

2.3. Characterisation of films

2.3.1. Mechanical properties

Resistance to fracture and deformability of films was determined using a TA.TXT*plus* Texture Analyser (Stable Micro Systems, Surrey, UK) as previously described [24]. The equipment was used in compression mode loaded with a 5 mm circular head probe in order to puncture at a speed of 0.5 mm/s a film secured in perpendicular position. The force and distance registered at film breakage were considered as the burst strength (resistance to fracture) and the elasticity (deformability). Films were further evaluated for pliability as detailed in the *Supporting Information* (*S1. Supporting methods*).

2.3.2. Polymer-plasticiser interactions

Raw materials and films were characterised by Fourier Transform Infrared Attenuated Total Reflection Spectroscopy (FTIR-ATR) using a Perkin-Elmer spectrophotometer equipped with a MIRacle™ accessory (Perkin-Elmer, Waltham, MA, USA). Spectra were obtained by conducting ten runs per sample over the wavelength range of 600-4000 cm^{-1}.

Table 1. Composition of films prepared, expressed as mg/film.

Batch	ERS	ERL	ES	EL	Glycerol	PEG	TBC	TEC	Oleic acid	Lactic acid
ERS G20	250				50					
ERS G40	250				100					
ERS G60	250				150					
ERS G80	250				200					
ERS TBC20	250						50			
ERS TBC40	250						100			
ERS TBC60	250						150			
ERS TBC80	250						200			
ERS OA20	250								50	
ERS OA40	250								100	
ERS OA60	250								150	
ERS OA80	250								200	
ERL G20		250			50					
ERL G40		250			100					
ERL G60		250			150					
ERL G80		250			200					
ERL TBC20		250					50			
ERL TBC40		250					100			
ERL TBC60		250					150			
ERL TBC80		250					200			
ERL OA20		250							50	
ERL OA40		250							100	
ERL OA60		250							150	
ERL OA80		250							200	
ES PEG20			250			50				
ES PEG40			250			100				
ES PEG60			250			150				
ES PEG80			250			200				
ES TEC20			250					50		
ES TEC40			250					100		
ES TEC60			250					150		
ES TEC80			250					200		
ES LA20			250							50
ES LA40			250							100
ES LA60			250							150
ES LA80			250							200
EL PEG20				250		50				
EL PEG40				250		100				
EL PEG60				250		150				
EL PEG80				250		200				
EL TEC20				250				50		
EL TEC40				250				100		
EL TEC60				250				150		
EL TEC80				250				200		
EL LA20				250						50
EL LA40				250						100
EL LA60				250						150
EL LA80				250						200

2.3.3. Drug release

The release profiles of TFV from vaginal films were evaluated as previously described [20, 25]. Briefly, individual films (loaded with 30mg TFV) were emerged in a 100 mL borosilicate glass bottle containing 80 mL of dissolution medium. Bottles were placed on a thermostatised shaking water bath at 15 opm and at 37 °C. Five millilitre aliquots were collected periodically and replaced with fresh medium. The amount of TFV was then quantified by UV spectrophotometry at 260 nm. Films were evaluated in triplicate using SVF (pH 4.2) [26] and a mixture of SVF with SSF [15] in a ratio 1:4 (v/v) and final pH of 7.5 (which simulates conditions in the vagina after ejaculation [16]), as release medium. Sink conditions were maintained throughout all experiments. Drug release profiles were fitted to different mathematical models commonly used to assess drug release mechanisms, namely zero order, first order, Higuchi, Hixson-Crowell, Hopfenberg, Weibull, and Ritger-Peppas [27-29]. Details on each model are included in *Supplementary Information* (*S1. Supporting methods*).

Drug release profiles from different film formulations or among media were also compared by calculating the similarity factor (f_2) according to the following equation:

$$f_2 = 50\ log\left\{\left[1 + \left(\frac{1}{n}\right)\sum_{j=1}^{n}\left|R_j - T_j\right|^2\right]^{-0.5}\ x\ 100\right\} \quad \text{(Eq. 1)}$$

where n indicates the number of samples of each test and R_j and T_j are the amounts of drug released at each time for the reference and the test batch, respectively. A value of f_2 lower than 50 indicates a statistically significant difference among the drug release profiles compared [30].

2.3.4. Swelling behaviour

The swelling and erosion of films in considered media (SVF and SVF/SSF) were studied using the methodology originally described by Ruiz-Caro *et al*. [31] adapted for films [20]. In brief, films were placed over a stainless-steel disc and immersed in the medium. At predetermined time points, films were collected, blotted in filter paper to remove medium excess, and weighed. The swelling ratio (SR) percentage was determined according to:

$$SR = \left(\frac{F_w - F_d}{F_d}\right)\ \% \quad \text{(Eq. 2)}$$

where F_d and F_w correspond to the weight of the film before and after emersion in medium, respectively.

2.3.5. Microscopic morphology

The surface morphology of films was characterised using scanning electron microscopy (SEM) with a JEOL JSM-6335F microscope (JEOL Ltd., Tokyo, Japan) at 100x, 500x and 1000x magnifications, and an accelerating voltage of 15 V. The samples were sputter coated with gold for 90 s using a sputter module under high vacuum atmosphere.

2.3.6. Cytotoxicity

The *in vitro* toxicity of excipients, free TFV and films was evaluated using HeLa and Ca Ski cervical cells, and HEC-1-A endometrial cells acquired from ATCC (Manassas, VA, USA). HeLa, Ca Ski and HEC-1-A cells were maintained in DMEM, RPMI 1640 and McCoy's 5A media, respectively, supplemented with 10% FBS, 100 U/mL penicillin and 0.1 mg/mL streptomycin. Cells were maintained under standard conditions (37 °C, 5% CO_2 and 95% humidity). Media were replaced every 2-3 days [32].

Samples to be tested for toxicity were prepared differently: excipients and TFV were dissolved/dispersed directly in medium in the concentration range of 0.001 to 10 mg/mL, while

film extracts were prepared as previously described [33, 34] by adapting the methodology of ISO 10993-5:2009 (Biological Evaluation of Medical Devices). Briefly, films were immersed in medium for 24 h, at 37 °C and 100 rpm, at a ratio of 1 mL medium/cm^2 film. Obtained samples were used as such (100%) or diluted with medium to 50% and 10% (v/v).

Cytotoxicity of samples was determined using the resazurin reduction assay [35]. Cells were seeded in 96-well plates at 5000 cells per well and left under standard conditions for 24 h. Samples and positive (medium) and negative (1% Triton X-100 in medium) controls were incubated with cells for an additional 24 h. Cells were then washed twice with phosphate buffered saline (pH 7.4) before being incubated for 4 h at standard culture conditions with 1% resazurin (w/v). Fluorescence intensity resulting from the metabolism of resazurin by viable cells was measured at an excitation wavelength of 530 nm and an emission wavelength of 590 nm with a microplate reader. Values were normalized to the positive control (100% viability). All controls and samples were evaluated in triplicate. Cell viability vs. concentration plots were fitted using log-logistic regression in order to calculate half-maximal cytotoxic concentration (CC_{50}) values.

2.3.7. Drug permeability and membrane retention

The permeability of TFV was evaluated using two cell monolayer models based on either Ca Ski cells or HEC-1-A cells [36, 37]. The amount of drug retained (or associated) with cell monolayers was also quantified. Cell monolayers were obtained by seeding cells at a density of 3 x 10^5 cells/cm^2 on Millicell® cell culture inserts with 1 µm pore and area of 1.1 cm^2 (Merck-millipore, Billerica, MA, USA) that were pre-coated with rat tail collagen type I. Cells were maintained for 7-8 days in culture in order to allow monolayer formation, and the transepithelial electrical resistance (TEER) was determined periodically using an EVOM voltohmmeter with "chopstick" electrodes (World Precision Instruments, Sarasota, FL, USA).

Drug permeability and retention experiments were conducted by placing 0.16 cm^2 film samples (corresponding to 300 µg of TFV) in 0.5 mL of HBSS in the apical side of cell monolayers, and incubating at 37 °C and 100 rpm. Control experiments were also performed using 300 µg of free TFV dissolved in HBSS. Samples of 0.5 mL were collected periodically from 30 min to 4 h, and replaced by fresh HBSS [36]. Then, membranes containing cell monolayers were collected and washed with PBS, before TFV being extracted with dimethyl sulfoxide. The amount of drug in all samples was determined by UV spectrophotometry at 260 nm. All samples were tested in triplicate. The apparent permeability coefficient (P_{app}) of TFV was calculated from permeability profiles according to:

$$P_{app} = \frac{Q}{A \cdot C \cdot t} \quad \text{(Eq. 3)}$$

where Q is the total amount of TFV permeated (µg), A is the area available for permeation (cm^2), C is the initial concentration of drug in the apical side (µg/mL) and t is the time of the experiment (s) [37].

2.3.7.1. Statistical analysis

Two-way ANOVA with Dunnett's post-hoc test was used for performing multiple comparisons, namely for P_{app} values or drug associated with cell monolayers for different formulations and different cell monolayer models, using Prism® v. 5.03 (GraphPad Software, La Jolla, CA, USA). Values of $p<0.05$ were considered as denoting significance. All results are presented as mean ± standard deviation (SD) from three independent experiments, unless otherwise mentioned.

3. RESULTS AND DISCUSSION

3.1. Preliminary studies and optimisation of films

We conducted an initial series of experiments in order to assess the ability of different Eudragit® types to produce viable films. In this part of the work, TFV was not incorporated in any of the films. The work included the evaluation of different solvents (acetone, ethanol, methanol and isopropanol) and plasticisers (PEG, glycerol, lactic acid, oleic acid, TBC and TEC) regarding their ability to be used for solvent casting. Organoleptic, pliability and mechanical properties were assessed at this preliminary stage. Isopropanol was selected as a common solvent for all Eudragit® types. PEG, lactic acid and TEC were selected for obtaining ES and EL films, while glycerol, oleic acid and TBC were the best plasticisers for preparing ERS and ERL films, respectively. Details on these initial studies are included as *Supporting Information* (*S2.1. Influence of solvent on films properties* & *S2.2. Influence of plasticisers on films properties*).

Initial plasticiser screening was performed at a fixed amount of 40% relative to the amount of Eudragit®. However, this fixed amount was shown not to be suitable for all Eudragit®/plasticiser pairs, as evaluated by the pliability of films (*Supporting Information*, *S2.2. Influence of plasticisers on films properties*). Thus, additional optimization was further conducted based on the assessment of mechanical properties. Resistance and elasticity of films were evaluated by determining values for burst strength and distance at burst. A minimum strength of 5 N was considered as acceptable in order to attest the ability of films to withstand typical handling during manufacturing, transport, storage and vaginal administration. If this threshold was not achievable, the formulation with higher resistance for plasticiser was considered for subsequent experiments.

ERS originated films with the best mechanical properties in the absence of plasticisers (*Supporting Information*, *S2.2. Influence of plasticisers on films properties*). Such properties were just barely improved when plasticising agent were included, which was usually only possible in small amounts (Figure 1A-B). For example, only 20% TBC originated suitable films. Glycerol was only possible to be incorporated up to 40%. Oleic acid was the only one able to be used up to 80%, but with decreasing resistance and deformability starting at 40% levels. For these reasons, 20% plasticiser was selected for all ERS films.

Regarding films with ERL, even small amounts of glycerol improved deformability, while increasing levels reduced burst strength (Figure 1C-D). ERL G40 films were selected due to their ability to maintain resistance at forces above 5 N. TBC at 40% yielded plastic ERL films, while rubbery systems were formed at levels of 60-80%. Thus, ERL TBC20 films were selected as appropriate. Finally, ERL films with oleic acid showed an abrupt decrease in burst strength without improvement of elasticity when 40% or more plasticised was incorporated, justifying the choice for ERL OA20 films. Films of ES had similar behaviour, but values for burst strength and distance at burst were kept at stable for 60-80% levels of plasticiser (Figure 1E-F). Films with higher amounts of PEG were rubbery and could not be analysed. A previous study testing Eudragit® films incorporating PEG at 25% and 50% levels also denoted increasing deformability with greater amounts of plasticiser [38]. Taken together, ES PEG40, ES TEC40 and ES LA60 films were selected for further testing.

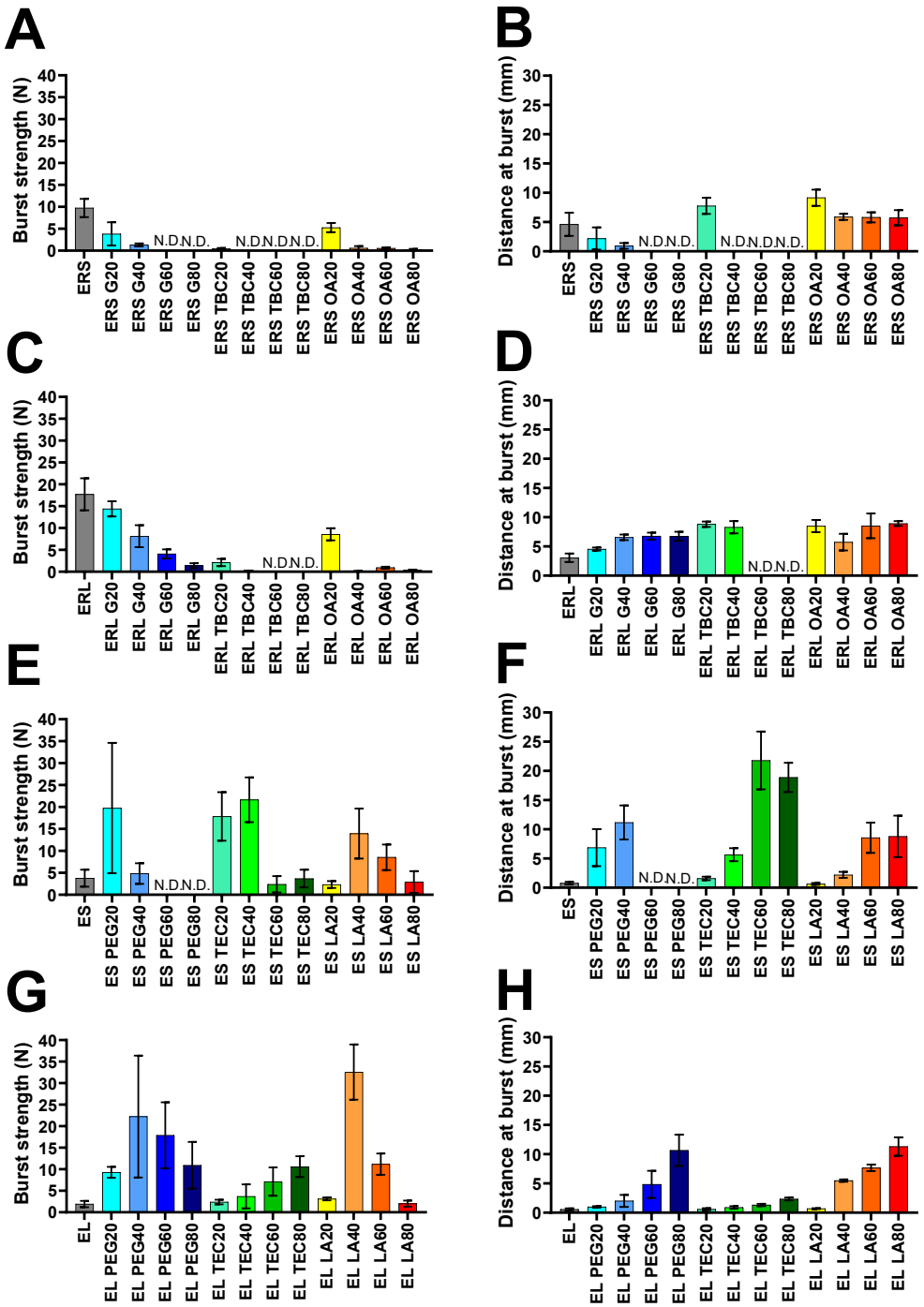

Figure 1. Film burst strength, expressed as the force (N) required to cause breakage, and distance at burst, denoting the deformation distance (mm) of films before rupture. Results are presented as mean ± SD (*n*=6).

Burst strength for EL films increased with higher amounts of TEC, thus justifying the choice for EL TEC80 films (Figure 1G). EL films became easily deformable for 80% levels of PEG and lactic acid, and EL PEG60 and EL LA60 were selected for additional characterisation. In all cases, distance at burst was increased with increasing amounts of plasticiser (Figure 1H).

Pliability of films proved the transition to rubbery films when some plasticisers are incorporated at higher concentrations. Moreover, films based on ES and EL required higher amounts of plasticiser to get suitable mechanical properties (*Supporting Information, S2.3. Influence of different amounts of plasticisers on pliability*). Finally, the characterisation by FTIR-ATR reveals that interaction polymer-plasticiser occurs by physical interaction except for films based on EL and ES plasticised with lactic acid or TEC, where chemical modifications seem to be present (*Supporting Information, S2.4. Interaction between film ingredients*). Overall, films used for incorporating TFV and included for the next round of testing were: ERS G20, ERS TBC20, ERS OA20, ERL G40, ERL TBC20, ERL OA20, EL PEG60, EL TEC80, EL LA60, ES PEG40, ES TEC40 and ES LA60.

3.2. TFV release from films

The twelve film formulations selected according to their favourable mechanical properties were used to incorporate 30 mg of TFV per film (2.1 mg/cm^2). The drug release profile in SVF (pH 4.2) and in a mixture of SVF/SSF (pH 7.5) was further evaluated in order to determine which Eudragit®-based formulation could better yield a dual drug release profile compatible with "smart" microbicides. Ideally, films should release minimal amounts of TFV while in acidic vaginal fluid, but allow accelerated drug release when semen (main vehicle for HIV) is deposited in the vagina. Drug release results in SVF for films based on ERS were considerably affected by the relative hydrophilicity of the plasticiser (Figure 2A). Glycerol is mixable with water and allows the film to become rapidly wetted by SVF, thus creating the possibility for TFV to be released almost completely from the insoluble ERS matrix within 24 h. On the contrary, more hydrophobic plasticisers like TBC or oleic acid were able to delay drug release in a sustained manner for up to 96-120 h. Even so, such waterproofing effect cannot be fully explained by the relative hydrophilicity of plasticisers since the considerable differences in aqueous solubility of TBC and oleic acid did not translate into different drug release profiles. Contrasting with ERS-based formulations, the release profile of TFV from ERL films was not influenced by plasticisers (Figure 2B). In this case, all films appeared to be highly permeable to SVF and rapidly released the drug within 5-6 h.

Films based on EL and ES featured slower drug release rates as compared to ERL and ERS films. Drug release from ES films denoted differences among plasticisers (Figure 2C). Again, highly hydrophilic materials such as lactic acid and PEG allowed faster release (near complete TFV released within 100 h) as compared to hydrophobic TEC (full release in roughly 200 h). A trend for early faster release in ES films plasticised with lactic acid as compared with those obtained with PEG was also noted. This effect was even more pronounced in the case of EL films (Figure 2D): incorporation of lactic acid and PEG resulted in complete TFV release within 100 h, while the inclusion of TEC extended this time window to roughly 300 h. These results are in line with our previous data showing that TEC can be used to not only improve the mechanical properties of Eudragit® films, but also achieve sustained release of hydrophilic drugs [39].

The values for f_2 comparing films based on the same Eudragit® type further denoted the existence of significant differences (*Supporting Information*, *Table S6*). ERS G20 films diverged from the other two matched ERS formulations, featuring faster TFV release. Differences among ERL films were only marginal between ERL G40 and ERL OA20 formulations. Significant differences between all EL films were observed, while only formulations containing TEC and lactic acid differed in the case of ES films.

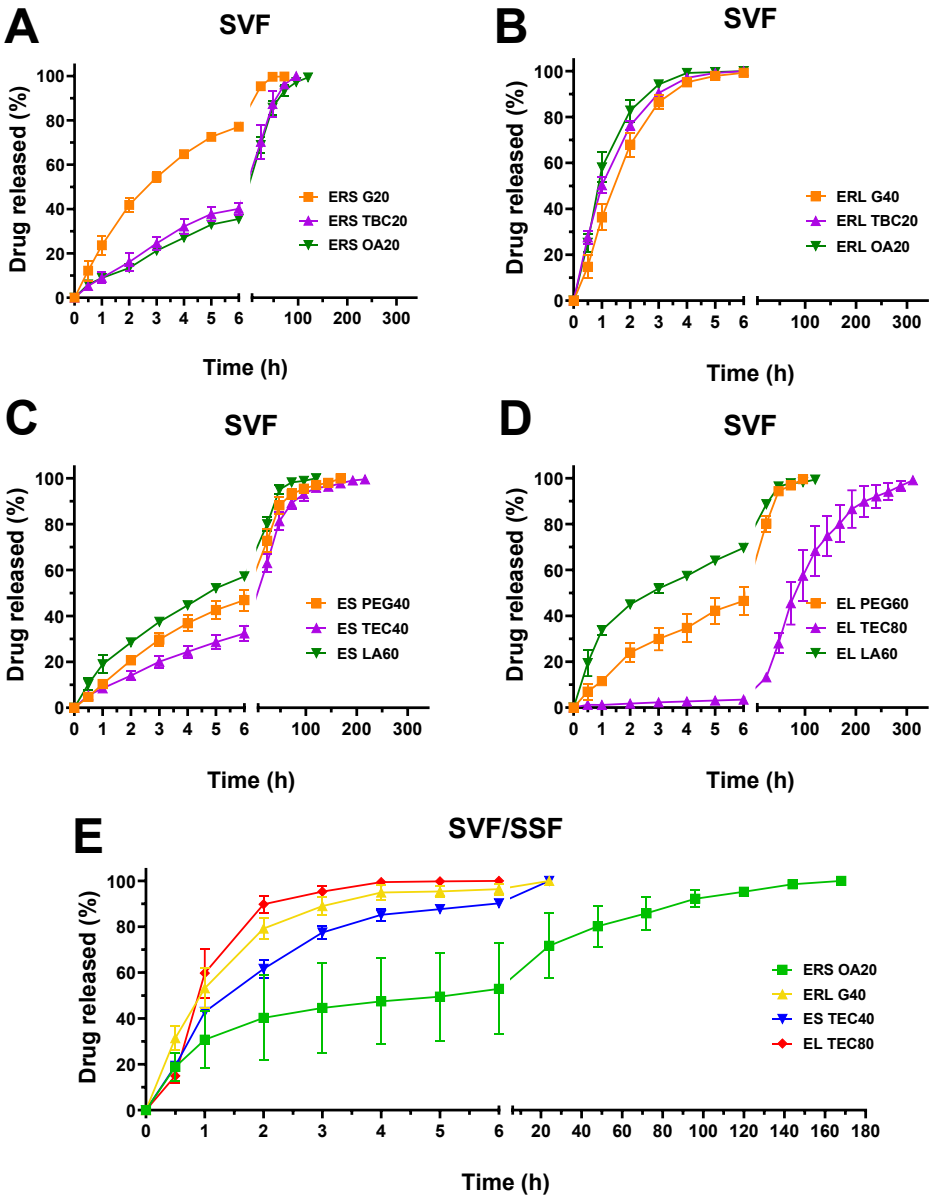

Figure 2. Drug release profiles in SVF, grouped by the Eudragit® type used for preparing films: ERS (a), ERL (b), EL (c) and ES (d). Drug release profiles of selected films in the mixture of SVF/SSF at ratio 1:4 (e). Results are presented as mean ± standard error of the mean (*n*=3).

Drug release data were further fitted to mathematical models commonly used in the evaluation of pharmaceutical dosage forms (*Supporting Information*, *Table S7*) [27-29]. Most films were well adjusted to the Higuchi model, thus denoting that diffusion is the main release mechanism of TFV. Overall good fitting to the Weibull model is also indicative of drug release from a matrix-type formulation. In addition, data from ERL films – which featured faster release of TFV in SVF – were well adjusted to Hixson-Crowell and Hopfenberg models. This indicates that structural modifications of ERL films resulting in matrix erosion are probably occurring. Good fitting to the Ritger-Peppas model with values of *n* generally between 0.5 and 1 (anomalous transport) indicates mixed diffusion and erosion as responsible for drug release. Since the polymer is not soluble at acidic pH, the observed erosion is likely due to the dissolution of the plasticiser in the medium, which leads to the onset of gaps within the polymeric matrix facilitating drug release.

The EL LA60 film deviated from the previous overall behaviour, with a value of *n* equal to 0.5. In this case, diffusion seems to be the only process that controls TFV release; this is consistent with poorer fittings of EL LA60 film data to the Hixson-Crowell and Hopfenberg models. Although based on limited experimental data (only two points of $Q_t < 0.6$), fitting results for ERL G40 and ERL OA20 films to the Ritger-Peppas model presented values of $n>1$ (which means a case II transport). This indicates that drug release may also be related to polymeric matrix relaxation, a phenomenon that has been previously reported for drug release from ERL and ERS matrix tablets [40]. The incorporation of water in ERL G40 and ERL OA20 films is presumably causing the relaxation of the polymer chains.

The film formulation among each Eudragit® type presenting a more sustained TFV release in SVF (ERS OA20, ERL G40, EL TEC80 and ES TEC40) were further evaluated in the presence of a mixture of SVF/SSF (1:4) in order to assess any pH-dependent dual drug release behaviour. Release of TFV from films based on ERL, ES and EL was quite fast (60-90% within 2 h) and nearly complete up to 6 h (Figure 2E). ERS OA20 films released the drug slowly and for over 150 h under slightly alkaline pH. This agrees with reports of the usefulness of ERS for producing matrix tablets that feature sustained drug release even at pH 7.2 [41].

We compared the release profiles of TFV in SVF and SVF/SSF in order to assess the usefulness of each film as pH-dependent dosage forms. Table 2 presents a summary of the time required for complete drug release in each medium, as well as f_2 values. Profiles significantly differed for all film formulations (f_2<50). Considerably faster release of TFV in SVF/SSF for ES TEC40 and EL TEC80 films supports that these systems may be useful as semen-triggered "smart" microbicide formulations. Although ERL G40 films features marginally different profiles in different media, the release of TFV was still considerably fast in SVF. This limits the possibility of providing pH-dependent effective dual drug release, but suggests that ERL films may still be useful as fast releasing microbicide formulations. In the case of ERS OA20 films, release of TFV was slow under both acidic and slightly alkaline conditions, which also undermines the initial purpose of this work. However, this suggests that ERS may be useful for developing sustained drug releasing products. A final technical note is deemed pertinent regarding the experimental setup used in our work. Performing independent drug release studies in SVF and SVF/SSF does not mimic the typical sequential exposure of films to different fluids upon vaginal administration. In particular, the release of TFV from films in SVF/SSF may be underestimated. However, we chose not to perform sequential drug release studies (i.e. the same film is placed initially in SVF and then in SVF/SSF) since transfer of films between containers with different media was not possible without compromising film integrity. Alternatively, addition of SSF to a film previously placed in SVF could be useful but the relative contribution of volume increase to the overall release profile could not be estimated.

Table 2. Comparison between complete drug release time (over 90% drug released) and f_2 values obtained in SVF and in SVF/SSF 1:4 for optimized films based on a specific Eudragit® type.

Films	Release time in SVF (h)	Release time in SVF/SSF (h)	f_2
ERS OA20	72	96	36.9
ERL G40	4	4	44.1
ES TEC40	96	6	18.6
EL TEC80	240	3	13.6

3.3. Swelling behaviour of films

We next conducted swelling tests using ERS OA20, ERL G40, EL TEC80 and ES TEC40 films in order to provide additional insights into drug release data. When immersed in SVF, liquid uptake was negligible for all formulations except for ERL G40 films (Figure 3A). The behaviour for this last film helps explaining its fast drug release in SVF, being consistent with considerable structural modifications of the polymeric matrix as suggested by the good fitting to Hixson-Crowell, Hopfenberg and Ritger-Peppas models [40]. The substantial water uptake leads to the relaxation of the structure of the film and allows fast release of TFV. As for ERS OA20, EL TEC80 and ES TEC40 films, maintenance of their weight suggests that no notable structural changes occurred in the polymeric matrix.

Another interesting observation is that films manufactured with the ES and EL underwent a quick loss of mass in SVF/SSF due to complete dissolution of the film (Figure 3B). This is consistent with the pH-dependent solubility profile of these Eudragit® types (soluble above pH 5.5 – EL – and above pH 7.0 – ES –, according to the specifications of the manufacturer) [23]. Again, this behaviour is consistent with the burst release of TFV from EL TEC80 and ES TEC40 films in SVF/SSF medium. Curiously, only minor to moderate fluid uptake was observed for ERS OA20 and ERL G40 films. These polymers are described by the manufacturer as insoluble, with pH-independent swelling, and low – ERS – or high permeability – ERL –. This test confirms the higher permeability of ERL at acid pH, but their swelling is clearly modified according to the pH of the medium, which could be associated to the presence of plasticisers.

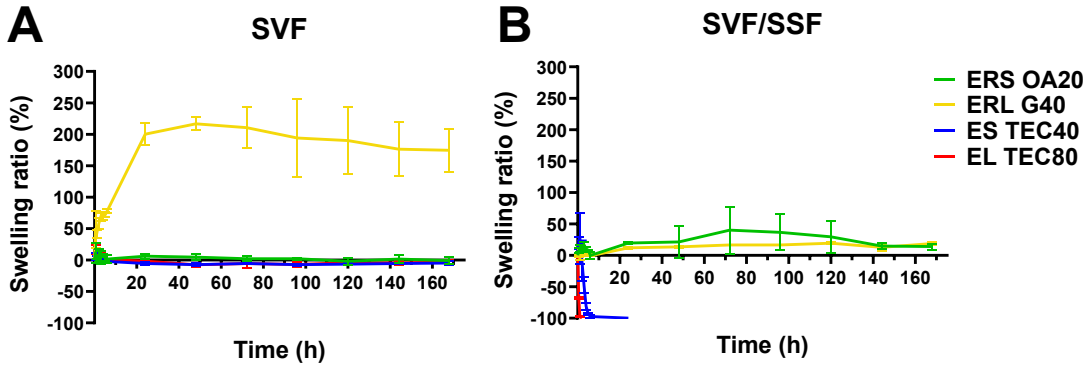

Figure 3. Comparison of swelling profiles of optimized films based on a specific Eudragit® type in SVF (a) and in the mixture SVF/SSF 1:4 (b). Results are presented as mean ± SD (*n*=3).

3.4. Surface morphology of films

Optimized films (ERS OA20, ERL G40, ES TEC40 and EL TEC80) containing TFV were observed by SEM in order to compare their surface appearance. Films prepared with ERS or ERL had a smooth surface, without observable pores (*Supporting Information, Figure S9*). Scattered crystals, likely from drug, were observed, and had homogeneous distribution along the surface of the film. This was expectable according to a previous study developing films containing these Eudragit® types [42]. The absence of pores may be relevant in reducing fast drug release. Films prepared with ES or EL presented porous surface, which was more pronounced in the case of the former. However, the effect of these pores on drug release was reduced due to the drug release mechanism of pH-sensitive Eudragit® [23]. This was confirmed by the results obtained previously for drug release and in swelling tests.

3.5. Cytotoxicity of films

The ability of different types of Eudragit® to prepare TFV-loaded films with different drug release profiles, motivated us to proceed with biological characterization pertaining to their tentative use as vaginal microbicides. Assessment of the safety potential of microbicides is paramount even at early development stages [43]. Cytotoxicity studies were initially conducted for individual excipients and TFV using three female genital cell lines relevant to vaginal drug delivery [44]. Values of CC_{50} are presented in Table 3 and full viability vs. concentration profiles are included as *Supplementary Information* (*Figure S10*). Results for TFV are in accordance with previous reports [44, 45] and support its relatively high safety profile (CC_{50} values typically above 1 mg/mL). Tested Eudragit® types featured overall low toxic potential, even if CC_{50} values for EL were lower. These differences could be due to the higher solubility of EL at the pH of cell culture media (pH 7.0-7.4). The higher amount of methacrylic acid in EL as compared to ES may also have led to decreased pH of cell culture media and, thus, mildly comprise cell viability. As for plasticisers, glycerol featured low toxicity, while oleic acid and TEC already denoted some deleterious effects at the low milligram per millilitre range. CC_{50} values for plasticisers are in line with those previously reported [39, 44, 46].

Table 3. Values of CC_{50} of raw materials included on films, calculated from data on HeLa, CaSki and HEC-1-A cells.

	CC_{50} (mg/mL)		
	HeLa	Ca Ski	HEC-1-A
TFV	6.7	2.7	2.5
Oleic acid	1.2	0.2	1.6
Glycerol	> 10	> 10	> 10
TEC	3.4	8.1	5.4
EL	3.9	3.0	9.1
ES	> 10	> 10	> 10
ERL	> 10	> 10	> 10
ERS	> 10	> 10	> 10

Cytotoxicity results of film extracts are presented in Figure 4 and correlated well with the toxicity of raw materials incorporated in each formulation. EL films were more toxic than formulations manufactured with other Eudragit® types. Additionally, plasticisers also contributed to observed cytotoxicity. ERL G40 films were safer than all other formulations when considering HeLa and Ca Ski cells, which could be correlated with the safety of glycerol. In the case of HEC-1-A cells, ES TEC40 films were the safest, despite containing the relatively more toxic plasticiser TEC. Deleterious effects arising from plasticisers were particularly apparent for ERS OA20 films, for which 70% or higher cell viability was only observed for extract dilutions at 50% (HeLa and Ca Ski cells) or 10% (HEC-1-A cells). Even so, the lower amount of plasticiser in ERS OA20 films still accounts for results that are roughly comparable to those of ES TEC40 films. The high content in plasticiser also seemed to contribute to the poorest safety profile of EL TEC80 film. The possibility of pH changes to cell culture media (rather than intrinsic toxicity of materials/film formulation) being responsible for observed results was discarded. For example, although ES and EL induced a more pronounced decrease in the pH of media – associated with the higher

content in methacrylic acid –, the pH of both extracts was similar (Figure 4). The cytotoxicity of the commercially available VCF® Vaginal Contraceptive Film (Apothecus, Oyster Bay, NY, USA) was also determined for comparison purposes. All Eudragit®-based films were shown safer than VCF®.

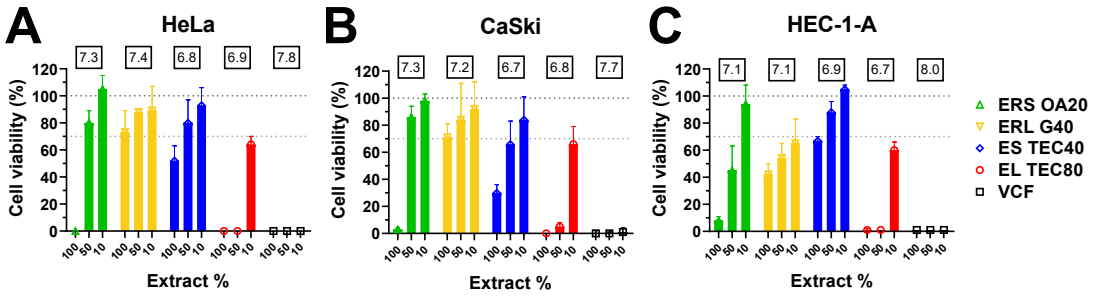

Figure 4. Toxicity potential of different film extracts to HeLa (a), Ca Ski (b) and HEC-1-A (c) cells. Cell viability was determined using the resazurin reduction assay and film extracts were tested without dilution (100%) or diluted to 50% and 10% in media. Values for pH of extracts without any dilution are presented over the dataset for each film. Results are presented as mean ± SD (*n*=3).

3.6. Permeability and membrane retention of TFV incorporated in films

The two most promising pH-triggered film formulations, ES TEC40 and EL TEC80, were further tested for their ability to influence the permeability and membrane association/retention of TFV using two cell monolayer models relevant to vaginal drug delivery [36, 37]. The permeability profile provides a proxy for the ability of a drug to penetrate the vaginal mucosa and eventually reach systemic circulation, while quantification of the drug associated with the cell monolayer allows estimating the potential for accumulation at the epithelial barrier. Permeability profiles are showed in Figure 5A-B. Free TFV allowed permeability to occur faster and to a mildly greater extent as compared to films, which is denoted by the significantly different values obtained for P_{app} (Figure 5C). The need for TFV to be initially released from films, as observed in drug release studies, creates a time gap between the beginning of the experiments and the time that the drug is actually available for starting to permeate the cell monolayers. Such an effect can be deemed beneficial not only in decreasing unintended systemic exposure to the drug, but also in providing sustained levels of TFV in the vagina [47]. It further implies that ES TEC40 and EL TEC80 films are unlikely to have caused notable damage to the cell monolayer barrier, thus backing-up the safety of tested formulations. Although providing similar permeability ranking regarding the free drug and films, the Ca Ski cell model presented higher absolute permeability to TFV as compared to the HEC-1-A model. This is in accordance with a previous study from our group using such models [37], and denotes the leakier nature of Ca Ski cell monolayer barrier.

Furthermore, the amount of TFV associated with cell monolayers (either internalised or adsorbed at cell membranes) was similar for both films and free TFV (Figure 5D). These results indicate that the films do not interfere with the ability of TFV to accumulate at the epithelial barrier (where the first transmission steps of HIV-1 occur). Again, differences among cell monolayer were observed for TFV association, with slightly higher values being observed for the Ca Ski cell model. It should be noted that permeability experiments were conducted under conditions that are closer to the time when semen is present in the vagina due to the near neutral pH of media. Although drug permeability and retention behaviour of films at acidic pH could not be assessed without significantly affecting the cell models used, our results appear to

support that films can provide adequate vaginal levels of TFV at least during the period when the drug is most useful in terms of protection from HIV-1 transmission.

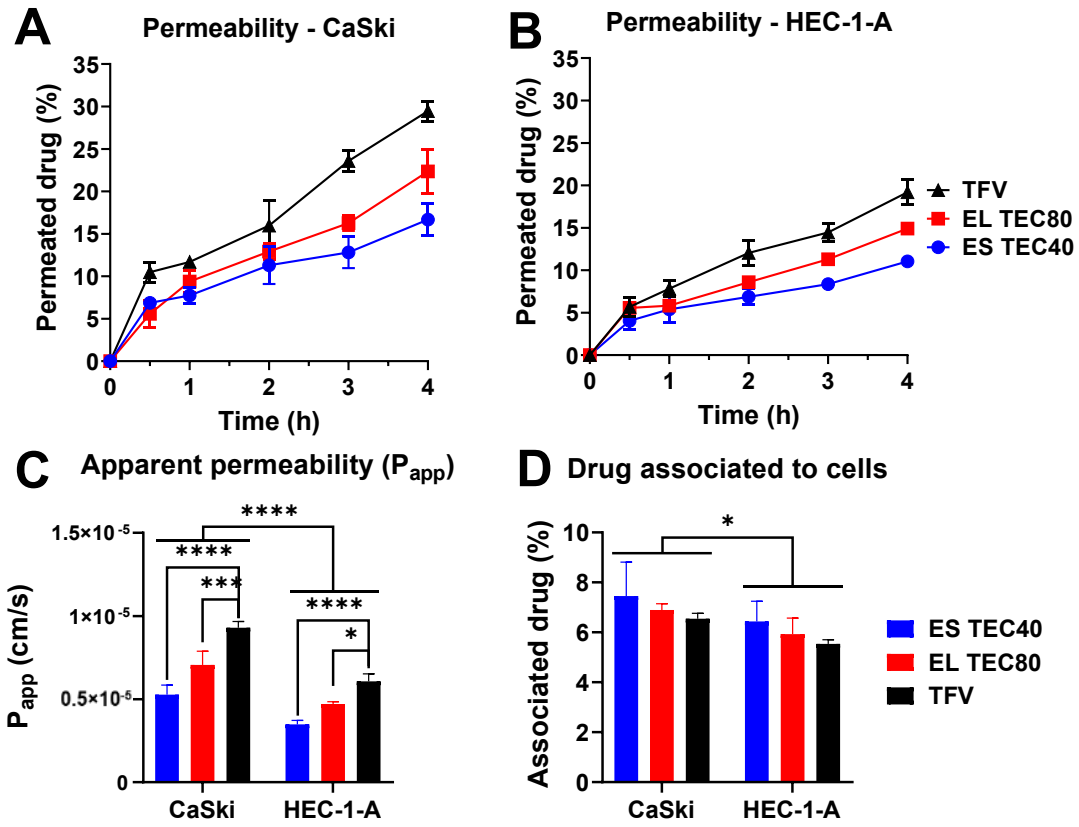

Figure 5. *In vitro* permeability and retention of TFV. Presented data include drug permeability across Ca Ski (a) and HEC-1-A (b) cell monolayers, calculated P_{app} values (c), and TFV associated with both type of cell monolayers after 4 h incubation (d) for the free drug, ES TEC40 and EL TEC80 films. Results are presented as mean ± SD (n=3). (*), (***) and (****) denote significant differences at p<0.05, p<0.001 and p<0.0001, respectively.

4. CONCLUSIONS

Formulations based on either EL and ES were shown particularly promising in sustaining TFV release under acid conditions, and providing fast drug release in slightly alkaline medium.

Proper qualitative and quantitative selection of plasticisers according to the type of Eudragit® used becomes essential for obtaining films with suitable organoleptic and mechanical properties for vaginal administration. Plasticisers were also shown relevant in determining the *in vitro* release of TFV from film.

Two formulations, namely ES TEC40 and EL TEC80 films, were shown moderately safe and able to provide similar permeability and epithelial retention levels of TFV. Overall, these two film formulations may constitute interesting candidate "smart" microbicide products that could enhance the protective potential of TFV in the context of vaginal HIV-1 transmission.

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AUTHOR CONTRIBUTIONS

Fernando Notario-Pérez: Conceptualization, Investigation, Writing – Original Draft. Joana Galante: Investigation. Araceli Martín-Illana: Investigation. Raúl Cazorla-Luna: Investigation. Bruno Sarmento: Conceptualization, Writing – Review & Editing, Supervision, Funding acquisition. Roberto Ruiz-Caro: Conceptualization, Writing – Review & Editing, Supervision. José das Neves: Conceptualization, Writing – Review & Editing, Supervision. María-Dolores Veiga: Conceptualization, Writing – Review & Editing, Supervision, Project administration, Funding acquisition. All the authors have approved the final article.

DECLARATIONS OF INTEREST

Declarations of interest: none

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Supporting Information

Development of pH-sensitive vaginal films based on methacrylate copolymers for topical HIV-1 pre-exposure prophylaxis

Fernando Notario-Pérez, Joana Galante, Araceli Martín-Illana, Raúl Cazorla-Luna, Bruno Sarmento, Roberto Ruiz-Caro, José das Neves, María-Dolores Veiga

S1. Supporting methods

S1.1. Pliability

Pliability provides organoleptic information on the ability of films to withstand folding [1] and was conducted according to Notario-Pérez *et al*. [2]. Briefly, films were manually folded in two equal parts and classified as (i) rigid (cannot be either broken – unless considerable force is applied – or folded), (ii) very fragile (are extremely fragile and cannot be removed from moulds without breaking), (iii) fragile (break when attempting to be folded), (iv) slightly flexible (can be moderately folded but not completely without breaking), (v) flexible (can be completely folded without breaking, but cannot recover its initial shape), and (vi) completely flexible (can be fully folded without breaking, and can recover their initial shape). An additional seventh category was included due to the properties of films presenting an excess of plasticiser: (vii) rubbery films were characterised by their stickiness and inability to maintain a defined shape. When handled, rubbery films were easily deformed and maintained the given shape upon rest.

S1.2. Mathematical models used for fitting drug release profiles

Drug release profiles were fitted to different mathematical models commonly used to assess drug release mechanisms, namely zero order, first order, Higuchi, Hixson-Crowell, Hopfenberg, Weibull, and Ritger-Peppas (Table S1) [3-5].

Table S1. Summary of mathematical models commonly used to evaluate drug release from dosage forms.

Model	Equation	Model description
Zero order	$Q_0 - Q_t = K_0 \cdot t$	Drug sustained release while the film remains unchanged.
First order	$\log Q_t = \log Q_0 - \frac{K_1 \cdot t}{2.303}$	Drug release as a function of surface action. Applied to porous matrices.
Higuchi	$Q_t = K_H t^{1/2}$	Drug release by a diffusion mechanism based on Fick's law.
Hixson-Crowell	${Q_0}^{1/3} - {Q_t}^{1/3} = K_{HC} \cdot t$	Drug release in parallel planes, the dimension is reduced but the size remains constant.

Hopfenberg	$\frac{Q_t}{Q_0} = 1 - [1 - k_{HF} \cdot t]^{n_H}$	Surface eroding dosage forms.
Weibull	$Q_t = Q_0 \left[1 - e^{-\frac{(t-t_{lag})^b}{a}}\right]$	Matrix-type systems.
Ritger-Peppas	$Q_t/Q_\infty = K_{RP} \cdot t^n$	Approximation of Korsmeyer-Peppas model. According to the value of n: Fickian diffusion model (n ≤ 0.5), anomalous transport – both diffusion an erosion – (0.5 < n < 1), zero order release (n = 1), case II transport (n > 1).

S2. Supporting results

S2.1. Influence of solvent on films properties

Organoleptic properties

A preliminary objective of the work was to determine the most appropriate solvent for obtaining Eudragit®-based films. Each polymer (ERS, ERL, ES and EL) was dissolved in 250 mg to 10 mL of acetone, ethanol, methanol or isopropanol and placed on moulds and allowed to dry completely. The macroscopic appearance of obtained films is presented in Figure S1. ERS in acetone and ERL in methanol yielded very fragile films featuring cracks resulting from demoulding. ES and EL in ethanol resulted in self-folded films, while ES and EL in acetone yielded heterogeneous films. Colouring of films was influenced by used solvent. For example, ES and EL in methanol resulted in whitish films, while the use of isopropanol led to transparent films. Different evaporation rates of these solvents lead to presumably different re-arrangement of polymer chains, as also reported for hydroxypropyl methylcellulose films dried at various temperatures [6]. Overall, films obtained using isopropanol were the most homogenous and transparent.

Pliability

Films need to be resistant to handling but still easy to fold in order to allow proper technological processing and suitable administration by women [7]. The results for pliability are shown in Table S2. Methanol and ethanol clearly failed to yield suitable films based on ES or EL. Conversely, acetone or isopropanol allowed obtaining slightly flexible films using the same polymers, even in the absence of plasticisers. Results for pliability of films prepared with ERS and ERL were different: ERS in acetone and ERL in methanol formed very fragile films that could not be unmoulded. Both polymers formed a flexible film when using isopropanol (even without a plasticiser), while ethanol led to films with excellent pliability, but with slightly plastic behaviour. Most of the batches prepared at this stage consisted of transparent or translucent films, which are generally preferred by women when considering vaginal use [8]. In view of these results, isopropanol was considered for further studies since it was the only one that allowed preparing suitable films using all tested Eudragit® types.

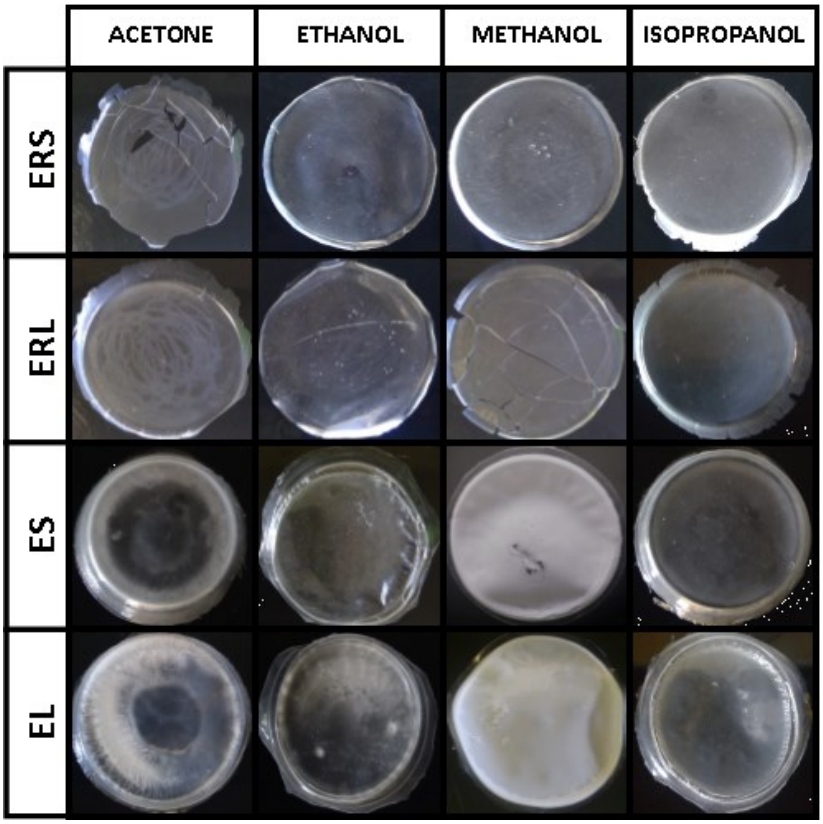

Figure S1. General appearance of films obtained with different Eudragit® types and solvents.

Table S2. Classification of films obtained with different solvents regarding pliability.

	Acetone	**Ethanol**	**Methanol**	**Isopropanol**
ERS	Very fragile	Flexible	Flexible	Flexible
ERL	Slightly flexible	Completely flexible	Very Fragile	Flexible
ES	Slightly flexible	Fragile	Fragile	Slightly flexible
EL	Slightly flexible	Fragile	Fragile	Slightly flexible

Mechanical properties

The mechanical properties of films were assessed by performing a puncture test using a Texture Analyser. The results for film burst strength and distance at burst are presented in Figure S2. Although mechanical properties seemed to be determined mostly with the Eudragit® type used, some variations were observed when using different solvents (Table S3). This was especially notable for films based on ERL; ethanol or isopropanol resulted in considerably higher burst strength and distance at burst as compared with acetone or methanol. These observations correlate well with pliability results. For the remaining Eudragit® types, There were no significant differences among the remaining films based on the same Eudragit® type but prepared with different solvents. ERS films stood out for their higher mechanical resistance, even in the absence of a plasticiser. Films based on ES featured the lowest flexibility, as noted previously by others [9]. These results further support the choice of isopropanol as solvent for preparing Eudragit® films. Also of notice, isopropanol is included in Class 3 (solvents with low toxic potential) of the Q3C(R6) ICH guidance on residual solvents in drug products [10].

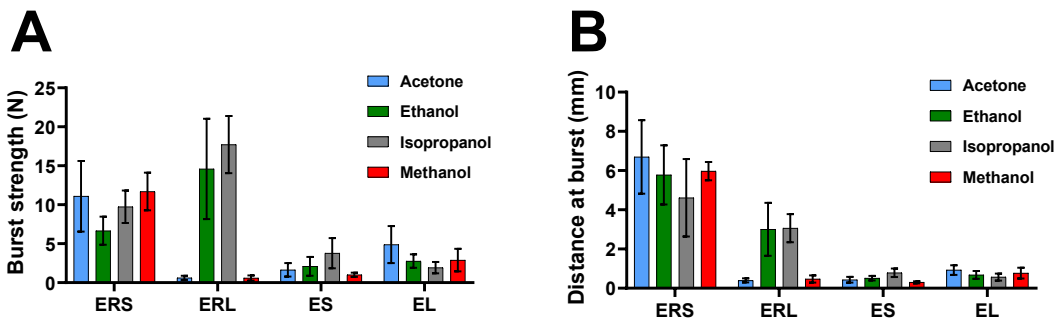

Figure S2. Results of film burst strength (A) and distance at burst (B) for films prepared with different solvents, expressed as the force (N) required to cause breakage and the stretch distance (mm) before breakage, respectively. Results are presented as mean ± SD (*n*=6).

Table S3. p-values obtained in multiple comparison by two-way ANOVA with Tukey's HSD post-hoc test. Values of p<0.05 were considered as denoting significance.

Comparison	Burst strength	Distance at burst
Eudragit® type	<0.0001	<0.0001
Solvent	<0.0001	0.1162
Interaction	<0.0001	<0.0001
ERS vs. ERL	0.2272	<0.0001
ERS vs. ES	<0.0001	<0.0001
ERS vs. EL	<0.0001	<0.0001
ERL vs. ES	<0.0001	<0.0001
ERL vs. EL	<0.0001	0.0011
ES vs. EL	0.5463	0.8112

S2.2. Influence of plasticisers on films properties

Organoleptic properties

Films based on Eudragit® and prepared with isopropanol were further optimized by testing the incorporation of different plasticisers. Polyethylene glycol (PEG), glycerol, lactic acid, oleic acid, tributyl citrate (TBC) and triethyl citrate (TEC) were selected [2, 11-13] and used at a fixed amount 40% (w/w) of the polymer (100 mg plasticiser/250 mg of Eudragit®). Organoleptic features of films were quite variable (Figure S3). Films based on ERS and ERL became sticky and plastic, especially in the presence of PEG, lactic acid or TEC. These films were highly deformable and lost their circular shape upon demoulding. Conversely, films prepared with ES or EL seemed to be more resilient, less flexible, and homogeneous (except with the incorporation of glycerol).

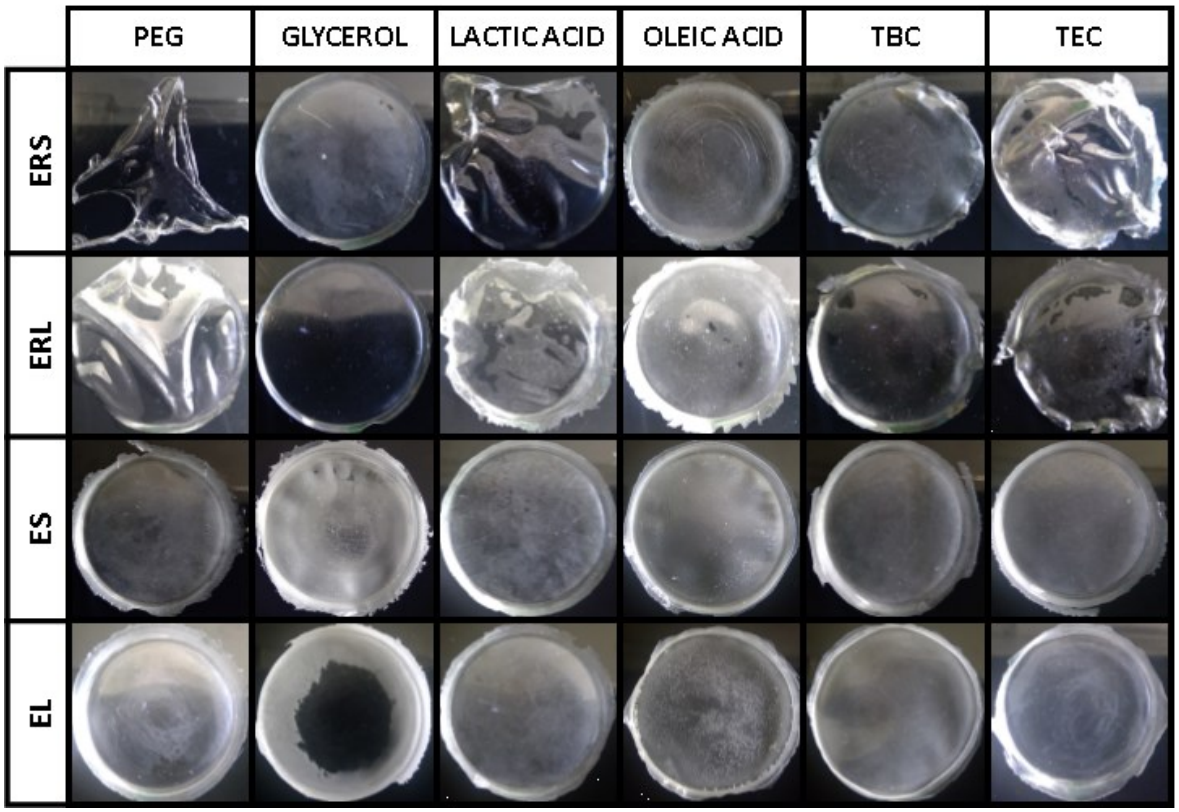

Figure S3. General appearance of Eudragit® films obtained using different plasticisers.

Pliability

Films based on ERS or ERL and using PEG, lactic acid or TEC as plasticisers were rubbery and deemed inappropriate for use (Table S4). ERS films also formed rubbery films with TBC, while ERL films prepared with TBC were elastic. Due to the similar nature of these last polymers, reducing the amount of TBC could potentially result in usable ERS films. ES and EL generally yielded acceptable films irrespective of the plasticiser that was used. Still, films based on EL were heterogeneous or fragile when incorporating glycerol or oleic acid, respectively.

Table S4. Pliability of Eudragit® films prepared with different plasticisers.

	PEG	**Glycerol**	**Lactic acid**	**Oleic acid**	**TBC**	**TEC**
ERS	Rubbery	Slightly flexible	Rubbery	Flexible	Rubbery	Rubbery
ERL	Rubbery	Flexible	Rubbery	Flexible	Flexible	Rubbery
ES	Flexible	Flexible	Slightly flexible	Slightly flexible	Slightly flexible	Slightly flexible
EL	Flexible	Flexible	Slightly flexible	Fragile	Slightly flexible	Slightly flexible

Mechanical properties

Films prepared with ERS or ERL and PEG, TEC or lactic acid were not evaluated for mechanical properties due to their rubbery behaviour (Figure S4A-D). ERS films with TBC were also not evaluated for the same reason. Plastic behaviour was generally increased by the incorporation of plasticisers, as evidenced by higher distance at burst as compared to corresponding plain Eudragit® films. This was particularly noticed in films with TBC and oleic acid, although similar effect was also apparent in films of ERL with glycerol. In addition, ERS films with glycerol featured decreased burst strength and distance at burst, which indicates an anti-plasticising effect due to an excess of plasticiser.

The mechanical properties for EL and ES films were substantially different (Figure S4E-H). Improved resistance and elasticity were noted when including lactic acid (especially when compared to films with oleic acid). This behaviour was also observed in ES films with TEC but not with TBC. EL and ES films with glycerol and PEG features enhanced mechanical properties over non-plasticised films. This effect was maximal in the case of ES films with PEG, and in line with the observations of Boateng *et al*. [9].

Taking in consideration the combined results for organoleptic, pliability and mechanical properties, glycerol, oleic acid and TBC were selected as the most suitable plasticisers for ERS or ERL films, while PEG, lactic acid and TEC appeared to be optimal for ES and EL films.

S2.3. Influence of different amounts of plasticisers on pliability

Contrasting with ES or EL films, which appeared to be able to incorporate 40% (w/w) of tested plasticisers without becoming greasy or rubbery, ERS and ERL films may require smaller amounts of these excipients in order to be usable. This possibility was tested by assessing the pliability of films when using the best plasticisers for each Eudragit® type at different percentages (Table S5). The behaviour of ERS films was variable for different amounts of plasticiser. Glycerol yielded fragile ERS films at 20%, which became greasy at 40%. The plasticiser was unable to be incorporated at 60% and over. In the case of ERS with oleic acid, films were flexible, although greater amounts of plasticiser resulted in more plastic and even greasier films. ERS films with TBC were rubbery at 40% of plasticiser, but became flexible at 20%. The behaviour for ERL films was similar. Flexibility was improved with higher amounts of the plasticiser, but became plastic and greasier at 60-80%.

The properties of ES films improved with increasing levels of plasticisers. However, lower amounts of PEG were required to improve flexibility as compared to films modified with lactic acid or TEC. A rubbery feeling was also noted for ES films with PEG at 60-80%. This ability of PEG to confer high flexibility to films is in agreement with a previous study on the development of composite hydroxypropyl methylcellulose/Eudragit® films [9]. Finally, the behaviour of EL films was similar to those of ES films.

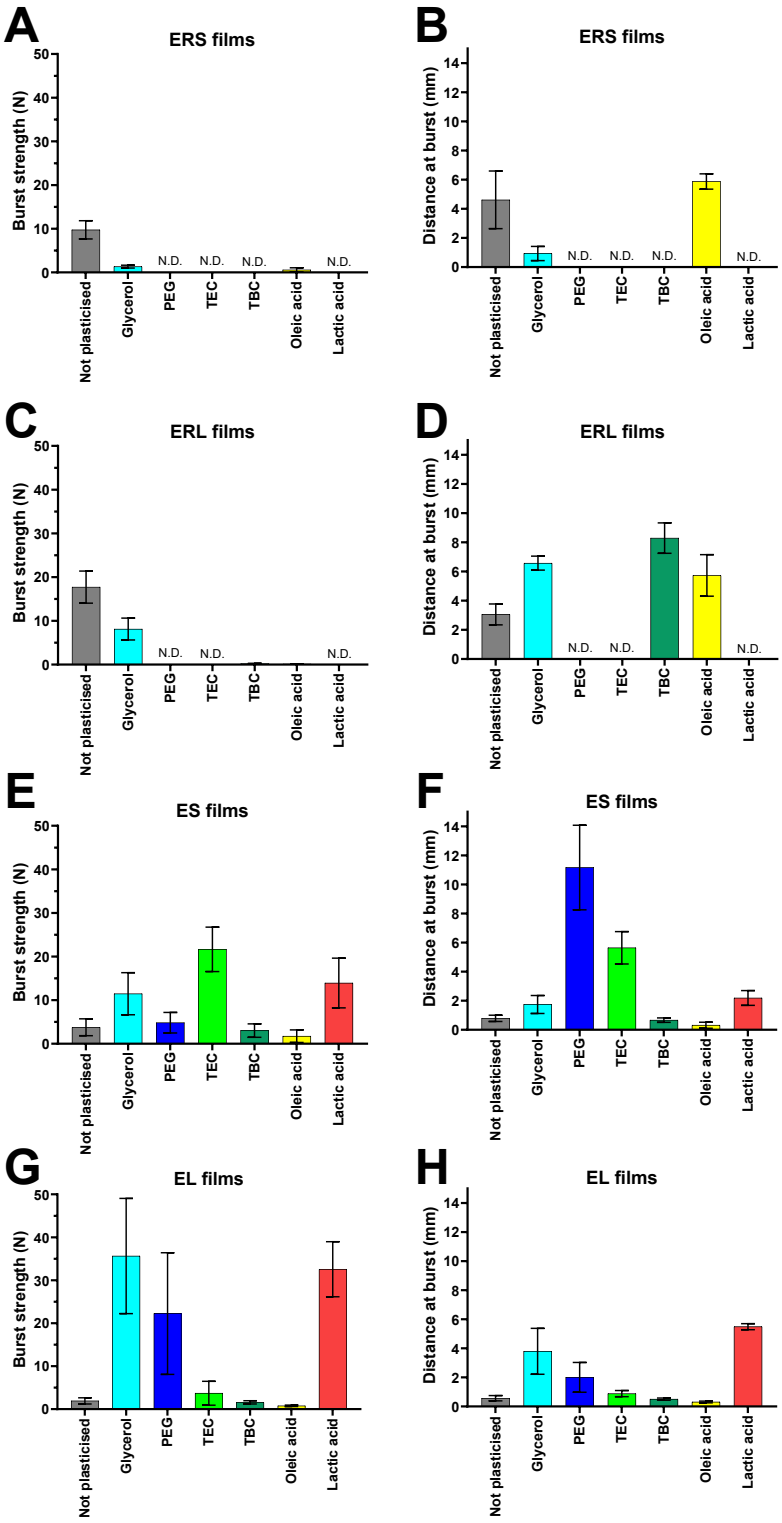

Figure S4. Results of the burst strength and distance at burst of films prepared with different plasticisers, expressed as force (N) required to cause breakage and the distance (mm) they are deformed before breaking, respectively. Results are presented as mean ± SD (*n*=6).

Table S5. Pliability of Eudragit® films prepared with different amounts of plasticisers (20, 40, 60 and 80% w/w regarding the amount of Eudragit®).

	20%	40%	60%	80%
ERS – Glycerol	Fragile	Slightly flexible	Plasticiser not incorporated	Plasticiser not incorporated
ERS – Oleic acid	Flexible	Flexible	Flexible	Flexible
ERS – TBC	Completely flexible	Rubbery	Rubbery	Rubbery
ERL – Glycerol	Flexible	Flexible	Completely flexible	Completely flexible
ERL – Oleic acid	Flexible	Flexible	Flexible	Flexible
ERL – TBC	Flexible	Flexible	Flexible	Flexible
ES - PEG	Slightly flexible	Flexible	Rubbery	Rubbery
ES – Lactic acid	Slightly flexible	Slightly flexible	Slightly flexible	Flexible
ES - TEC	Slightly flexible	Slightly flexible	Slightly flexible	Flexible
EL - PEG	Slightly flexible	Flexible	Completely flexible	Flexible
EL – Lactic acid	Fragile	Slightly flexible	Slightly flexible	Flexible
EL - TEC	Slightly flexible	Slightly flexible	Slightly flexible	Slightly flexible

S2.4. Interactions between film ingredients

The analysis by FTIR-ATR of films allows a better understanding of possible interactions among polymers and plasticisers, as well as to track for residual isopropanol in the films. Results indicate that the presence of the solvent is likely negligible, as denoted by the absence of its typical peaks at 3300 cm^{-1} (wide band corresponding to hydroxyl group) and 2900 cm^{-1} (associated to carbon-hydrogen stretching vibrations) (Figure S5-S8). Spectra of films with glycerol (Figure S5A and S6A) and PEG (Figure S7A and S8A) feature a peak at 3300 cm^{-1}, which can be attributed to the hydroxyl groups from these plasticisers rather than from residual isopropanol.

Spectra of films prepared with ERS or ERL showed no evidence of chemical modifications irrespective of the plasticiser (Figures S5 and S6). These observations support that plasticisation of ERS and ERL likely occurred through physical interactions, being consistent with the need for relatively lower amounts of plasticisers in order to obtain proper films. No chemical interactions were also noted in the case of films prepared with EL or ES and PEG (Figures S7A and S8A). Nevertheless, there was a potential interaction detected when lactic acid or TEC were used for obtaining EL or ES films: a wide band appeared at 1550-1650 cm^{-1}, which was more intense with higher amounts of plasticisers (Figures S7B-C and S8B-C). This peak is consistent with the presence of a carbonyl group from an amide or an ester [14, 15]. In films with lactic acid, the plasticiser could also be reacting with Eudragit® via free acid groups from both excipients, giving rise to acid polycondensation [16]. This peak is more apparent in films with EL (methacrylic acid/methyl methacrylate copolymer, 1:1), likely due to the higher content in methacrylic acid as compared to ES (methacrylic acid/methyl methacrylate copolymer, 1:2) [17]. Furthermore, this peak is also visible when TEC was incorporated into the films (Figures S7B and S8B). In this case, the ester groups of the plasticiser could be involved into a transesterification reaction with the methyl methacrylate groups from Eudragit®. The proportion of methyl methacrylate is higher in ES than in EL, and this may explain higher transesterification in films containing the former Eudragit® [17].

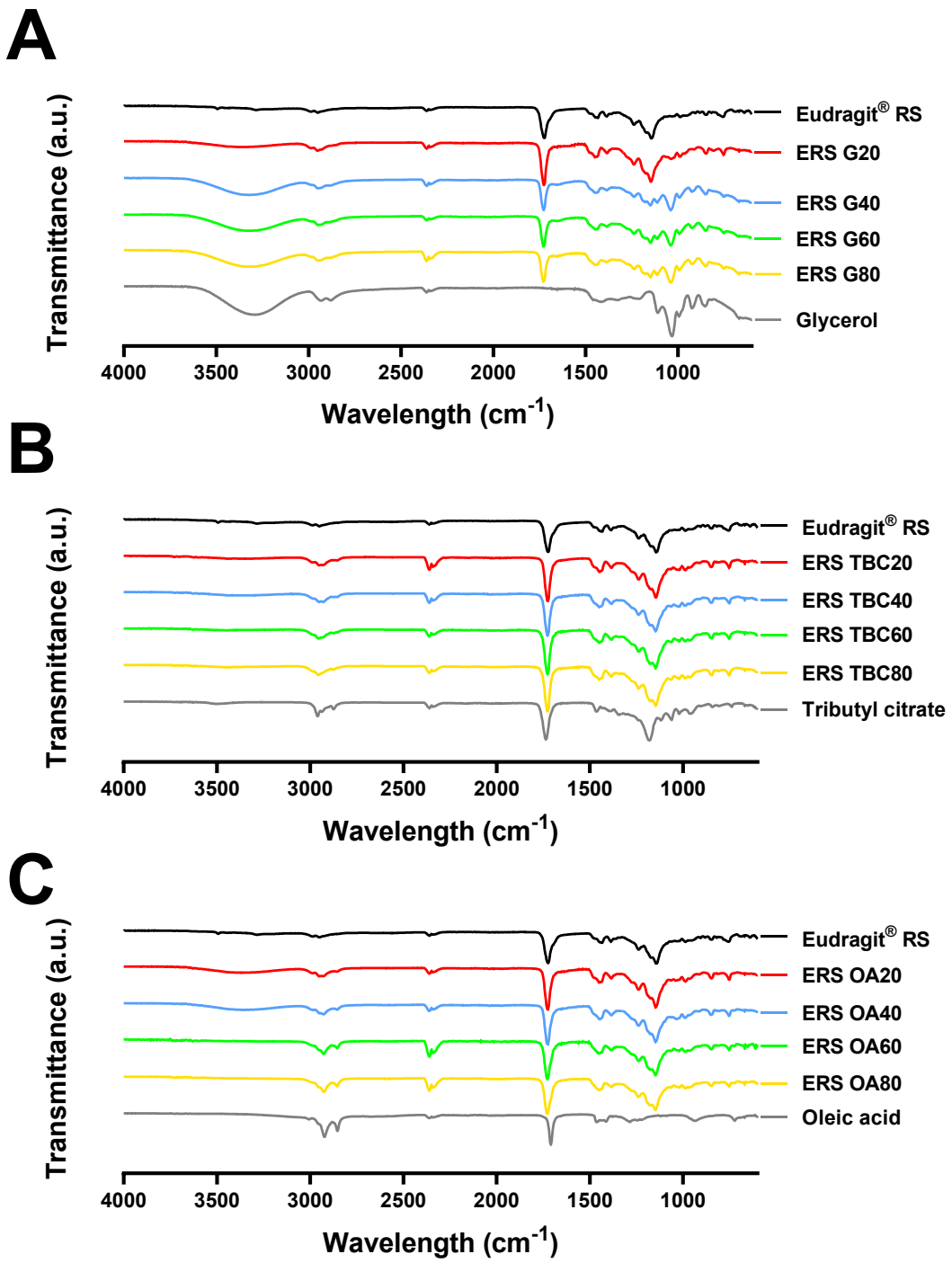

Figure S5. FTIR-ATR spectra of films of ERS with glycerol (A), TBC (B) or oleic acid (C). Numbers in front of each film indicate the percentage of plasticiser used.

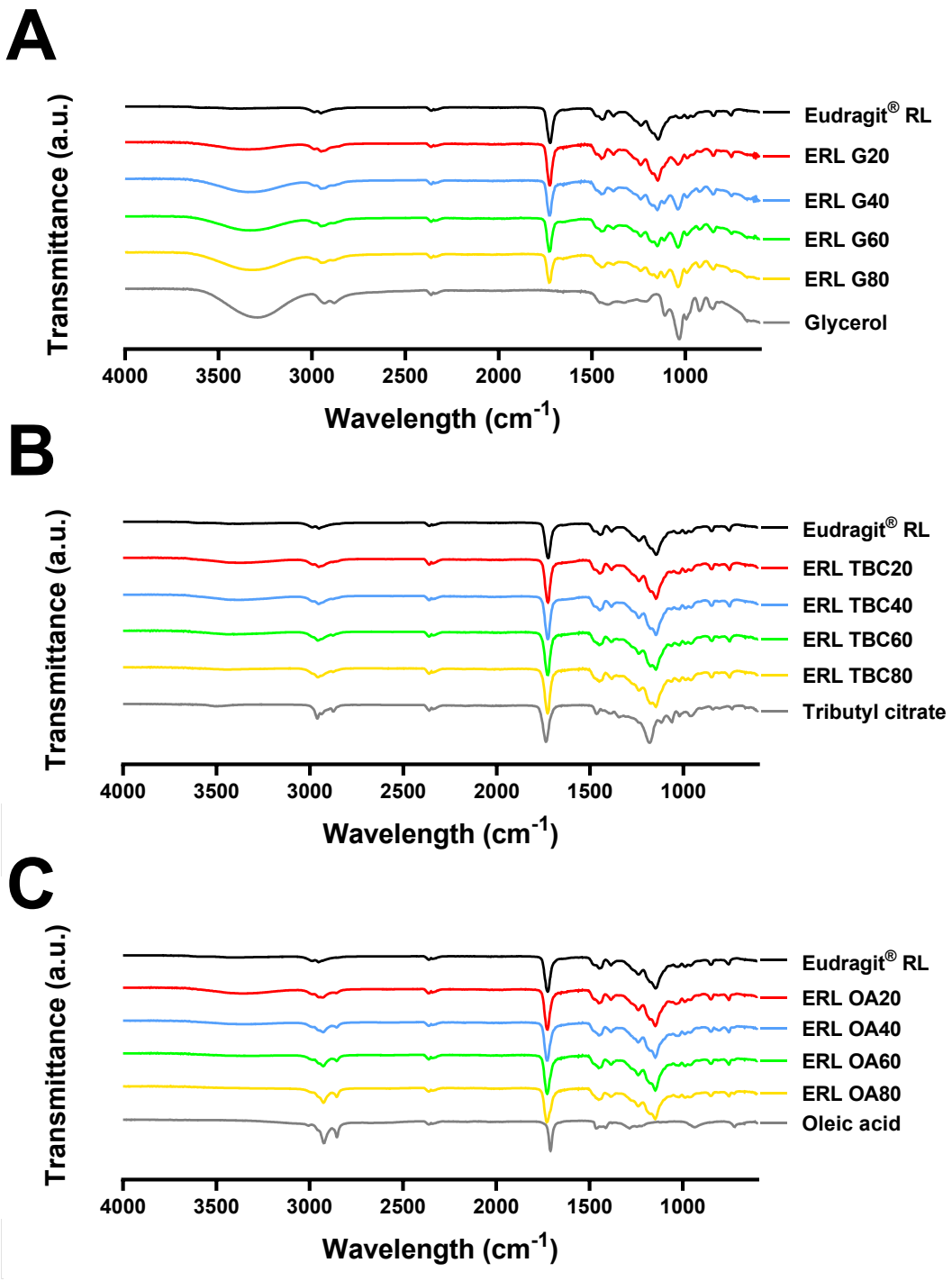

Figure S6. FTIR-ATR spectra of films of ERL with glycerol (A), TBC (B) or oleic acid (C). Numbers in front of each film indicate the percentage of plasticiser used.

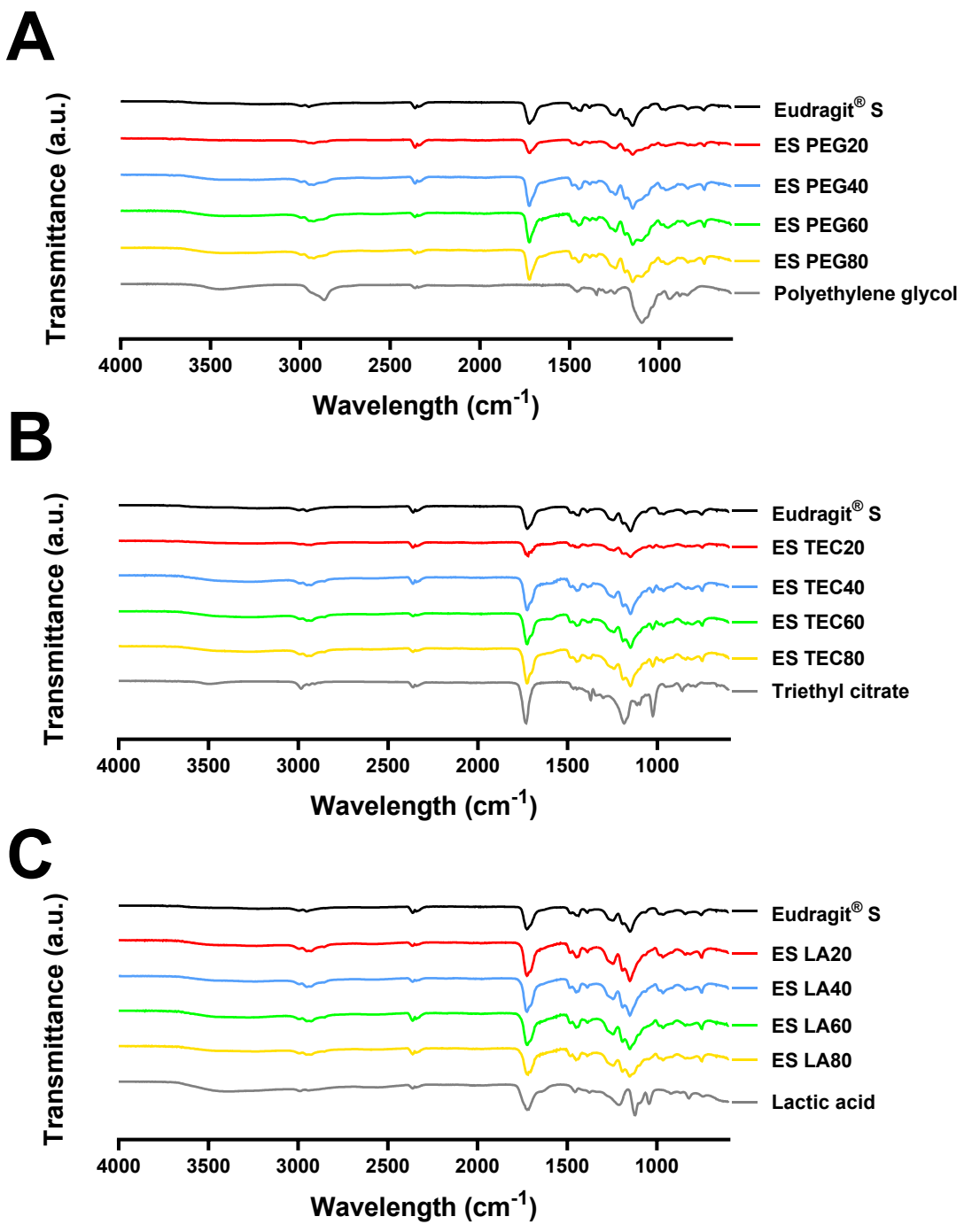

Figure S7. FTIR-ATR spectra of films of ES with PEG (A), TEC (B) or lactic acid (C). Numbers in front of each film indicate the percentage of plasticiser used.

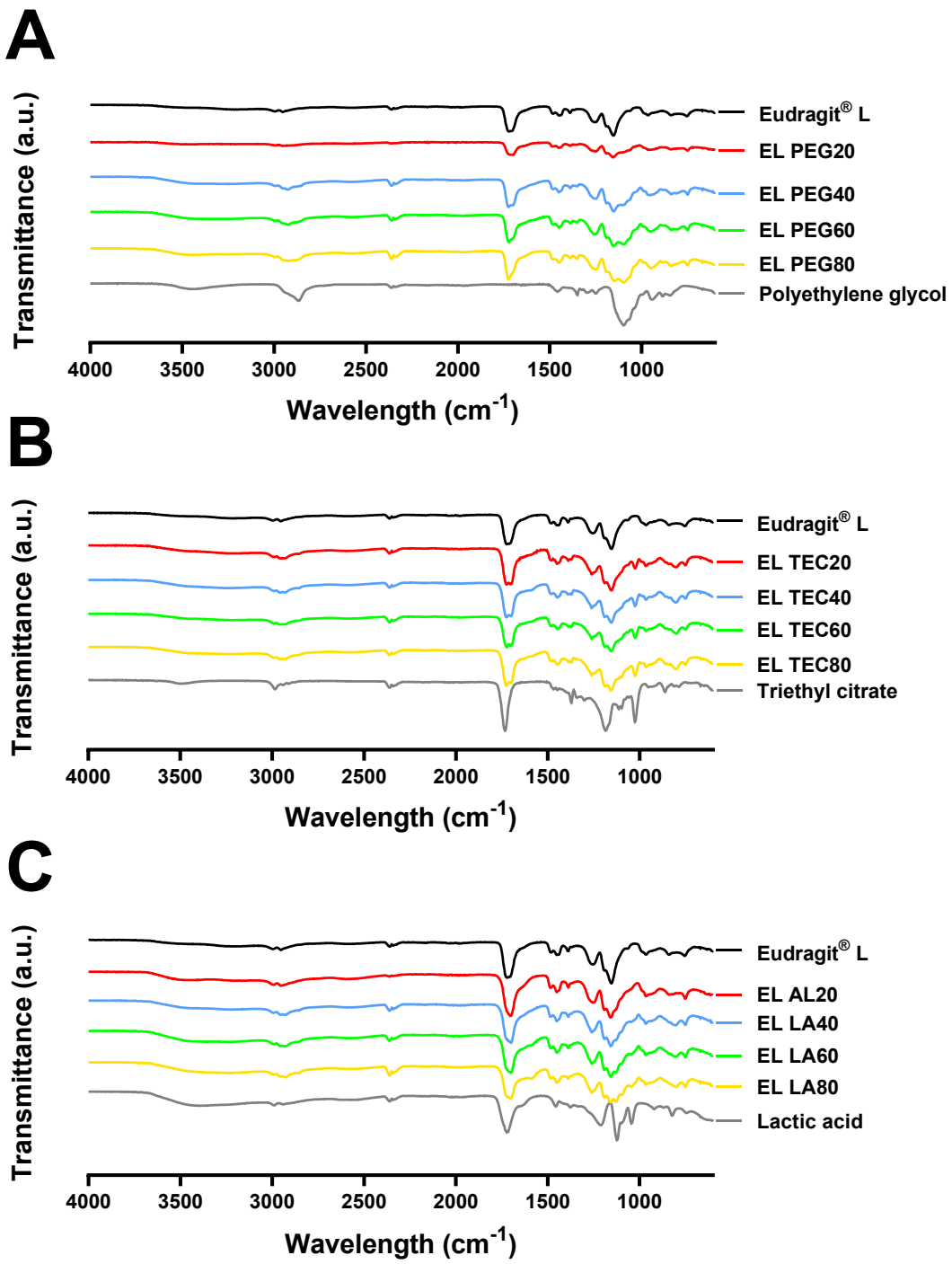

Figure S8. FTIR-ATR spectra of films of EL with PEG (A), TEC (B) or lactic acid (C). Numbers in front of each film indicate the percentage of plasticiser used.

S2.5. Drug release

Table S6. Similarity factor (f_2) values obtained from the comparison of drug release profiles in simulated vaginal fluid of reference and test formulations. Comparisons with significant difference (f_2 < 50) are in bold.

Film comparisons	f_2
ERS G20 *vs.* ERS TBC20	**29.1**
ERS G20 *vs.* ERS OA20	**26.9**
ERS TBC20 *vs.* ERS OA20	76.1
ERL G40 *vs.* ERL TBC20	51.9
ERL G40 *vs.* ERL OA20	**44.7**
ERL TBC20 *vs.* ERL OA20	64.6
EL PEG60 *vs.* EL TEC80	**20.8**
EL PEG60 *vs.* EL LA60	**36.0**
EL TEC80 *vs.* EL LA60	**14.9**
ES PEG40 *vs.* ES TEC40	52.0
ES PEG40 *vs.* ES LA60	55.8
ES TEC40 *vs.* ES LA60	**39.4**

Table S7. Correlation coefficients (r^2) and relevant constants obtained from fitting drug release profiles in simulated vaginal fluid to the different mathematical models. (*) denotes films for which only two points of Q_t < 0.6 were used and, thus, no representative correlation coefficients were possible to be calculated.

Sample	Zero order		First order		Higuchi		Hixson-Crowell		Hopfenberg		Ritger-Peppas			Weibull	
	r^2	K_0	r^2	K_1	r^2	K_H	r^2	K_{HC}	r^2	K_{HF}	r^2	K_{KP}	n	r^2	K_W
ERS G20	0.9495	0.128	0.8215	0.069	0.9820	0.343	0.9889	0.066	0.9814	0.088	0.9951	0.227	0.83	0.9979	0.251
ERS TBC20	0.8227	0.026	0.5212	0.185	0.9694	0.155	0.9103	0.013	0.8905	0.017	0.9964	0.093	0.85	0.9444	0.048
ERS OA20	0.8837	0.026	0.6106	0.187	0.9843	0.150	0.9507	0.013	0.9365	0.017	0.9871	0.094	0.74	0.9736	0.048
ERL G40	0.9963	0.348	0.9167	2.217	0.8978	0.474	0.9905	0.164	0.9939	0.225	*	0.367	1.29	0.9804	0.596
ERL TBC20	0.9643	0.373	0.9160	1.472	0.9768	0.538	0.9974	0.190	0.9927	0.254	*	0.503	0.86	0.9993	0.723
ERL OA20	0.9537	0.416	0.8351	1.686	0.9514	0.596	0.9902	0.227	0.9849	0.298	*	0.580	1.20	0.9918	0.908
EL PEG60	0.8258	0.030	0.5284	0.172	0.9786	0.174	0.9358	0.016	0.9125	0.021	0.9905	0.122	0.77	0.9714	0.065
EL TEC80	0.9855	0.005	0.8339	0.059	0.9699	0.068	0.9972	0.002	0.9964	0.003	0.9560	0.011	0.81	0.9931	0.010
EL LA60	0.8769	0.102	0.8159	0.447	0.9903	0.284	0.9409	0.049	0.9269	0.067	0.9652	0.305	0.50	0.9641	0.183
ES PEG40	0.7533	0.027	0.4423	0.178	0.9442	0.162	0.8614	0.013	0.8359	0.018	0.9909	0.099	0.92	0.9074	0.051
ES TEC40	0.8886	0.016	0.6174	0.100	0.9904	0.124	0.9618	0.008	0.9466	0.011	0.9986	0.088	0.73	0.9845	0.034
ES LA60	0.6905	0.028	0.4766	0.137	0.9242	0.173	0.8291	0.015	0.7957	0.020	0.9953	0.176	0.67	0.8896	0.062

S2.6. Surface morphology of films

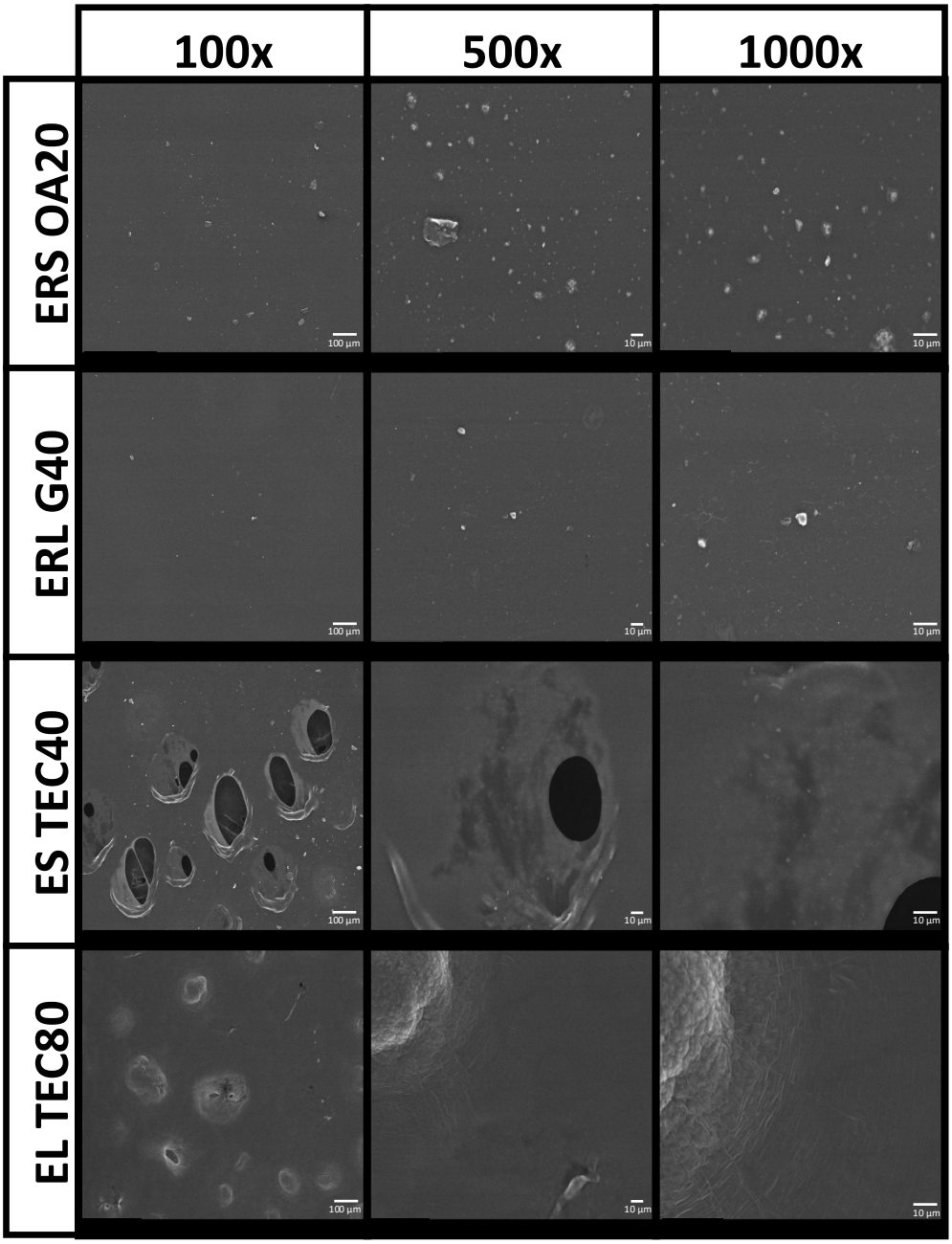

Figure S9. Scanning electron images of optimized films for each Eudragit® type. Microphotographs were taken with an acceleration voltage of 15 kV at a magnification of (left to right) 100x, 500x and 1000x.

S2.7. Cytotoxicity of individual film ingredients

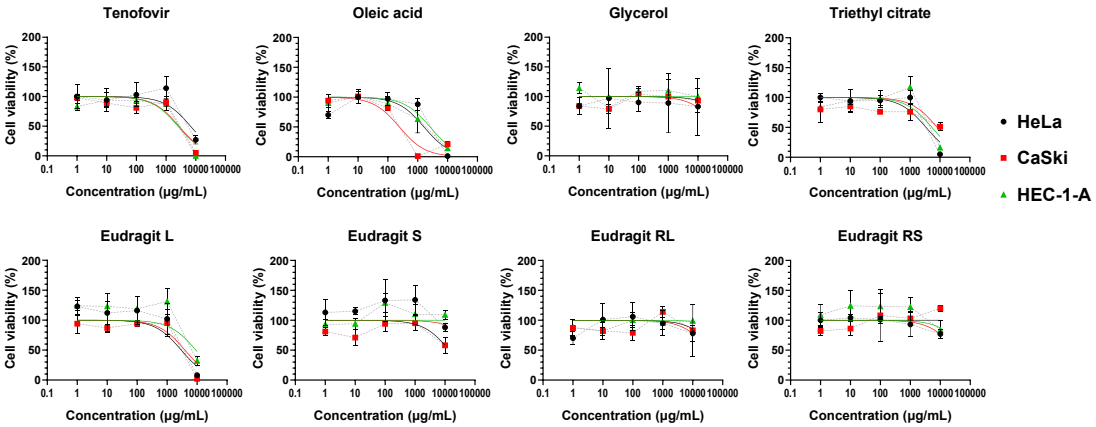

Figure S10. Cytotoxicity of raw materials used for film preparation. Viability of HeLa, Ca Ski and HEC-1-A cell lines was evaluated using the resazurin reduction assay. Results are presented as mean ± SD (*n*=3). Colorized continuous lines represent the log-logistic regression of experimental results.

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CAPÍTULO X

MUCOADHESIVE VAGINAL DISCS BASED ON CYCLODEXTRIN AND SURFACTANTS FOR THE CONTROLLED RELEASE OF ANTIRETROVIRAL DRUGS TO PREVENT THE SEXUAL TRANSMISSION OF HIV

pharmaceutics

MDPI

Article

Mucoadhesive Vaginal Discs based on Cyclodextrin and Surfactants for the Controlled Release of Antiretroviral Drugs to Prevent the Sexual Transmission of HIV

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Abstract: The strategies for developing vaginal microbicides to protect women against human immunodeficiency virus (HIV) sexual transmission are constantly changing. Although the initial dosage forms required daily administration to offer effective protection, the trend then moved towards sustained-release dosage forms that require less frequency of administration in order to improve women's compliance with the treatment. Nevertheless, another possible strategy is to design on-demand products that can be used in a coitally-dependent manner and only need to be administered immediately before intercourse to offer protection. Vaginal discs based on freeze-dried hydroxypropylmethyl cellulose gels have been developed for this purpose, containing two surfactants, i.e., sodium dodecyl sulphate and polysorbate 60, alone or in combination with 2-hydroxypropyl-β-cyclodextrin, to achieve a formulation capable of incorporating both hydrophilic and lipophilic drugs. Several studies have been carried out to evaluate how the inclusion of these substances modifies the structure of gels (viscosity and consistency studies) and the porosimetry of the freeze-dried discs (scanning electron microscopy micrographs, mechanical properties, swelling behaviour). The drug release and mucoadhesive properties of the discs have also been evaluated with a view to their clinical application. The systems combining sodium dodecyl sulphate and 2-hydroxypropyl-β-cyclodextrin were found to be adequate for the vaginal administration of both Tenofovir and Dapivirine and also offer excellent mucoadhesion to vaginal tissue; these discs could therefore be an interesting option for a coitally-dependent administration to protect women against HIV transmission.

Keywords: coitally-dependent microbicide; dapivirine; freeze-dried gels; human immunodeficiency virus; 2-hydroxypropyl-β-cyclodextrin; mucoadhesive vaginal discs; surfactant; tenofovir

1. Introduction

Human immunodeficiency virus (HIV) infection continues to be a major health problem worldwide, although in recent years great strides have been made in access to antiretroviral therapy and in slowing the incidence of new infections [1]. According to the latest data from the World Health Organization, in 2018 there were 37.9 million people living with HIV, of which 1.7 million were infected in that year. Women deserve a special mention, as it is estimated that every day about 860 young women (between

15–24 years old) become infected. These data are especially worrying in sub-Saharan Africa, where 80% of young people suffering from the infection are women [2].

Consequently, research into vaginal microbicides has soared in recent decades. These microbicides are formulations with a topical application that can be used by women without the need for men's cooperation. They need to be effective in preventing the transmission of the virus, safe (with a minimal effect on the integrity of the cervicovaginal epithelium), and comfortable for women [3,4]. Although numerous strategies were initially evaluated to develop vaginal microbicides (surfactants, acidifiers, polyanions, monoclonal antibodies, etc.), it has now been observed that only microbicides containing antiretroviral drugs can actually inhibit in vivo infection [4–6].

Undoubtedly, the two most widely studied antiretroviral drugs for the manufacture of vaginal microbicides are Tenofovir (TFV) and Dapivirine (DPV), given the good results obtained in clinical trials [5]. TFV is a nucleoside reverse transcriptase inhibitor (NRTI) that has been included as a microbicide in numerous pharmaceutical forms (gels, films, rings, tablets) [7,8]. It is notable that the first microbicide to demonstrate protective efficacy in clinical trials was a 1% TFV gel (CAPRISA 004 trial), which was shown to reduce HIV infections by 39% [9]. In contrast, DPV is a non-nucleoside reverse transcriptase inhibitor (NNRTI); it is a non-competitive inhibitor that binds to reverse transcriptase at an allosteric site and causes a conformational change in the enzyme [10]. Although films, gels, and tablets loaded with this drug have also been developed, vaginal rings are particularly worth highlighting, since two clinical trials conducted with this formulation (The Ring Study and ASPIRE trial) demonstrated protective efficacy against HIV [11,12]. Although the two drugs have proven potential, it should be noted that while TFV has demonstrated its efficacy in vaginal gels (as a hydrophilic drug, strategies have been sought to accelerate its dissolution after administration), vaginal rings have generally been chosen for long-term release in the case of DPV (made possible thanks to the lipophilicity of the drug, which is not very soluble in vaginal fluid). It is difficult to find a suitable formulation in which the two drugs could be incorporated, although some researchers have succeeded in developing both vaginal films and rings that combine the two active principles [13,14].

Among the different dosage forms developed for the vaginal administration of microbicides (gels, creams, tablets, films, and vaginal rings [3]), gels are undoubtedly the most frequently used, since they allow a rapid release of the active substance (which enables their use on demand prior to sexual intercourse), and they are also economical and easily for women to apply [5]. However, the main problems cited by users are leakage and messiness after administration [15]. Freeze-drying is one strategy to solve this problem with semi-solid formulations, involving a process in which water is removed from the pharmaceutical form [16,17]. These freeze-dried gels are equally convenient to administer and allow the rapid release of the active substance, since they can quickly rehydrate in the presence of vaginal fluid [18]. This would also make it possible to minimize and even eliminate the inconvenience caused by the leakage of the gels.

Gels for vaginal administration can be prepared in a wide variety of natural, synthetic or semi-synthetic polymers [19], among which it is worth highlighting cellulose derivatives for their extreme versatility. Since it is a semi-synthetic polymer, the cellulose can be modified by substituting different functional groups in order to obtain a wide variety of polymers (hydroxypropylmethyl cellulose (HPMC), methylcellulose, hydroxypropyl cellulose, hydroxyethyl cellulose, ethyl cellulose, etc.), all of which can be used to prepare vaginal gels, depending on the desired characteristics in the formulation [20–22].

When formulating gels with poorly water-soluble drugs such as DPV, excipients such as surfactants or cyclodextrins must be added to ensure the solubility of the drug in the aqueous medium. Cyclodextrins have been shown not only to increase drug solubility but also to have the potential to modulate the release rate of the active substance, thus offering a strategy for the development of sustained-release formulations [23]. It has also been observed that the structure of gels based on cellulose derivatives can be modified by the incorporation of cyclodextrins, which vary the

arrangement of the polymer network [24]. Finally, there are references to possible interactions when surfactants are combined with cyclodextrins [25].

Vaginal discs have been developed in this work. These discs consist of freeze-dried gels manufactured with a flat and circular shape. Thus, they provide all the previously mentioned advantages of freeze- dried systems for vaginal administration. Moreover, it must be mentioned that the shape of the formulation has been designed with a view of vaginal films, trying also to provide the advantages of this dosage form. The flat shape will provide a more comfortable administration, while the large surface would allow better mucoadhesion and faster water capture.

Based on this background, the aim of this work is to prepare vaginal discs obtained by freeze-drying gels based on HPMC. These gels incorporate a water-soluble antiretroviral drug (TFV) or a poorly water-soluble drug (DPV). Surfactants (both neutral and anionic in nature) and cyclodextrins will also be included to determine their possible influence on the gel structure, to determine how their presence affects freeze-dried gels, and particularly to assess their ability to modify the drug release. The objective is to develop a formulation that is comfortable for women to administer and that ensures the rapid dissolution of the drugs, therefore allowing their discreet use on demand immediately before sexual intercourse. Thus, the developed microbicide could improve the main problem observed in clinical trials with these drugs, which was the lack of adherence to treatment by women.

2. Materials and Methods

2.1. Materials

Tenofovir (TFV, lot: FT104801501) was provided by Carbosynth Limited (Berkshire, UK). Dapivirine (DPV; lot: 60416PIL04) was kindly provided by the International Partnership for Microbicides (IPM, Silver Spring, MD, USA). Hydroxypropylmethyl cellulose—Methocel®K4M (HPMC; lot: SK27012N11) was kindly supplied by Colorcon Ltd. (Kent, UK). 2-Hydroxypropyl-β-cyclodextrin (2HPβCD; lot: BCBQ9423V), Polysorbate—Tween®60 (P60; lot: MKBT3178V), and sodium dodecyl sulphate (SDS; lot: STBJ1530) were acquired from Sigma–Aldrich (St. Louis, MO, USA). Technical-grade water (lot: 9V011540) was acquired from Panreac (Barcelona, Spain).

All other reagents in this study were of analytical grade and were used without further purification. Demineralised water was used in all cases, except in the preparation of the gels, when technical-grade water was used.

2.2. Gel Preparation

Six batches of unloaded gels were manufactured. The reference batch was a 2% HPMC gel, prepared to evaluate the structure of the gel when no other component was added. Three HPMC-based gels including 10% of 2HPβCD, SDS (anionic surfactant) or P60 (neutral surfactant) were then manufactured in order to determine how the addition of these components modified the polymer network and gel properties. To evaluate possible interactions between surfactants and cyclodextrins, two extra HPMC gels were also prepared containing 10% of 2HPβCD and 10% of SDS or P60. Finally, the same six batches containing 0.25% TFV or 0.125% DPV were prepared as drug-loaded gels. The composition of the gels was proportional to the dose per disc desired, which is presented in Table 1.

Gels were prepared by dissolving the cyclodextrin and/or surfactant in double-distilled water, then adding the drug until its dissolution (or until its homogeneous dispersion in batches with DPV but without surfactant, when the active principle was not dissolved). Finally, HPMC was added, and the system was stirred until its complete gelation. The gels were left to rest for 72 h prior to their characterisation to guarantee their complete homogenization. A bulk gel of each batch was prepared and characterised. Subsequently, each vaginal disc of the same batch was prepared from this gel.

Table 1. Composition of vaginal discs (mg).

Group	Batch	Hydroxypropylmethyl Cellulose	2-Hydroxypropyl-β-Cyclodextrin	Sodium Dodecyl Sulphate	Polysorbate 60	Tenofovir	Dapivirine
Unloaded	H	80					
	HC	80	400				
	HS	80		400			
	HSC	80	400	400			
	HT	80			400		
	HTC	80	400		400		
TFV-loaded	H-TFV	80				10	
	HC-TFV	80	400			10	
	HS-TFV	80		400		10	
	HSC-TFV	80	400	400		10	
	HT-TFV	80			400	10	
	HTC-TFV	80	400		400	10	
DPV-loaded	H-DPV	80					5
	HC-DPV	80	400				5
	HS-DPV	80		400			5
	HSC-DPV	80	400	400			5
	HT-DPV	80			400		5
	HTC-DPV	80	400		400		5

2.3. Gel Characterisation

2.3.1. Viscosity

The viscosity of the gels was determined using a viscosimeter (Visco Elite, Fungilab S.A., Sant Feliu Llobregat, Spain): 100 mL of the gel at room temperature was placed in a 4 cm diameter tube. The viscosity (mPa·s) was measured every 10 s for 180 s, using the appropriate probe (L2, L3 or L4). However, the values were observed to be constant after the first 20 s, so it was decided to use values obtained after 30 s of the test. This study was carried out on the bulk gel prepared for each batch.

2.3.2. Penetration and Detachment Work

A study was carried out using a Texture Analyser (TA.XT*plus*, Stable Micro Systems, Surrey, UK) to determine the penetration work (a measure of gel consistency) and the detachment work (related to gel adhesiveness). The characterisation was done using the protocol of Martín-Illana et al., with a 2 cm diameter probe and a 5 kg load cell [17]. Specifically, 80 mL of the evaluated gel was placed in a 100 mL beaker. The probe was located 60 mm above the base of the equipment (10 mm above the gel surface) and was lowered onto the gel at a speed of 0.5 mm/s after an activation force of 2 g. In the test, the probe penetrated the gel to a distance of 1.5 cm and was then returned to its initial position. The force required by the probe to penetrate and detach the gel was recorded. The test was performed nine times for each batch. The results were compared with a paired *t*-test, with $\alpha = 0.05$.

2.4. Vaginal Disc Preparation

Vaginal discs were prepared by freeze-drying the previously prepared gels (Figure 1). The gels were dosed on silicone templates with a diameter of 5 cm in a sufficient amount to obtain a final dose of 10 mg TFV or 5 mg DPV for each disc. The amount of TFV was equivalent to the dose in gels and fast-release films already tested in clinical trials [9]. The amount of DPV included was higher than the dose required to achieve inhibitory concentrations, in order to characterise the amount of this drug that could be dissolved with the applied solubilization technologies.

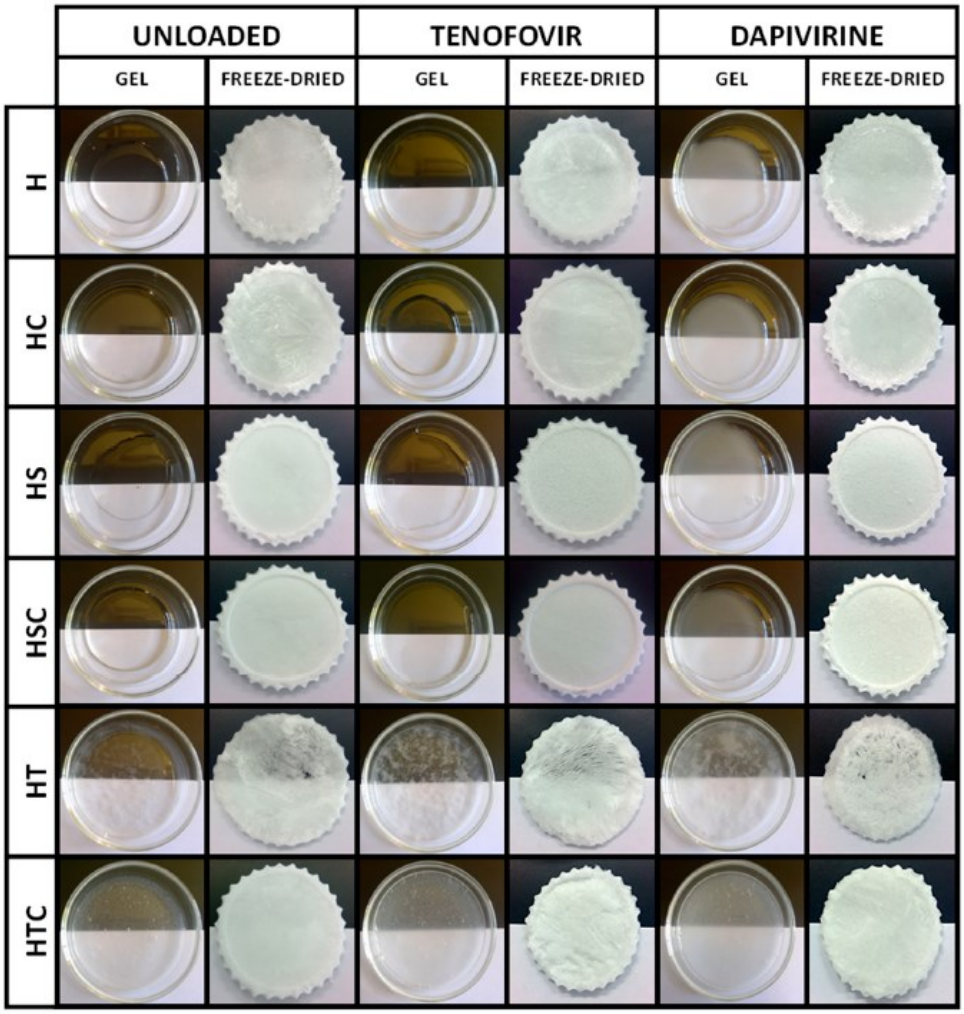

Figure 1. Appearance of each batch of fresh and freeze-dried gels.

Unloaded vaginal discs were also prepared by adding the required amount of gel to obtain discs with the same composition as their equivalent drug-loaded disc (except for the active principle, obviously). A Lio-Labor®freeze-dryer (Telstar, Barcelona, Spain) was used to obtain the discs, using a freezing temperature of –45 °C, a sublimation pressure of 4.5×10^{-4} atm and a sublimation temperature between –45 °C and 25 °C [17,26]. The composition of all the prepared batches is shown in Table 1. The visual appearance of the gels and discs can be seen in Figure 1.

2.5. *Vaginal Disc Characterisation*

2.5.1. Apparent Density Calculation

To evaluate the uniformity of each batch of vaginal discs, the discs were weighed on a precision balance and the height was measured with the Texture Analyser. This was done by placing the 2 cm-diameter probe above the vaginal disc at a distance of 5 cm and lowering it at a speed of 0.1 mm/s until a force of 10 g was detected, at which point it stopped. The height of the discs was determined as the difference between the distance of the probe at the start of the trial (5 cm) and the distance at which it stopped. Apparent density of the vaginal discs was determined from the disc diameter (5 cm) and the height values. These tests were done with eight samples of drug-loaded discs and five samples of unloaded discs. Since the discs have a cylindrical flat shape, Equation (1) was used to calculate their

apparent density, where ρ is the apparent density and m, r, and h represent the weight, the radius (25 mm), and the height of the disc, respectively.

$$\rho = \frac{m}{\pi \cdot r^2 \cdot h} \quad (1)$$

2.5.2. Porosity Measurement

A vaginal disc from each batch was also characterised by mercury porosimetry using an Autopore II 9215 (Micromeritics Corp., Norcross, GA, USA). Pose size distribution (PSD) was determined, and the corresponding mean pore size (Dp), pore volume (Vp), apparent density (ρ), and porosity (P) were calculated based on this, assuming the pores have a cylindrical shape [27]. Cumulative and incremental pore size distribution graphs were plotted with these data.

2.5.3. Scanning Electron Microscopy (SEM) Micrographs

Micrographs of the surface of the vaginal discs were captured using a scanning electron microscope (JEOL JSM-7600F, JEOL Ltd., Tokyo, Japan) at an accelerating voltage of 5 kV. One disc from each batch was fixed on the microscope sample holder and coated for 90 s in a high-vacuum atmosphere with a gold sputter module. Micrographs were captured at ×25, ×50 and ×100 magnifications.

2.5.4. Mechanical Properties: Deformation and Resistance to Fracture

These tests were done using the Texture Analyser. For the deformation test, the equipment was installed with a 5 kg load cell, and a 2 cm cylindrical probe was placed at an initial height of 5 cm. The vaginal disc was placed on the base of the Texture Analyser, and the probe was lowered onto the disc at a rate of 0.5 mm/s. When an activation force of 2 g was detected, the Texture Analyser began recording the force applied until the vaginal disc was deformed by 1 mm. The probe then returned to its initial position before descending again in a cyclical mode, until ten deformation cycles had been completed [18]. The test was performed with four samples of each batch. The deformation force was established as the force required to deform the disc by 1 mm in the first cycle, and the loss of deformability was calculated as the difference between the force applied in the first and the tenth cycle, as a percentage.

Elasticity and resistance to fracture of the discs were determined with a method previously used to evaluate vaginal films [28]. The puncture test was conducted with a probe with a circular head 5 mm in diameter. The film was fixed on a rigid support with a hole, and the probe was lowered through this hole at a speed of 0.5 mm/s. The force applied and the distance the disc was deformed were recorded, and the disc burst strength was established as the force at which the probe caused the disc to rupture. This test was also done in quadruplicate.

2.5.5. Swelling Behaviour

Once administered, the vaginal disc captures vaginal fluid to reconstitute the gel. To evaluate how this process takes place, swelling studies were done using simulated vaginal fluid (SVF), prepared as described by Owen and Katz [29]. The vaginal discs were fixed to stainless steel discs using an acrylic adhesive and placed at the bottom of a 250 mL beaker, immersed in SVF. The beakers were inserted in an oscillating water bath (Selecta®Unitronic320 OR, Barcelona, Spain) at 37 °C and 15 opm to simulate conditions in the vaginal environment. At predetermined times—0.5, 1, 2, 3, 4, 5, 6, 24 h, and each 24 h from that point—the discs were removed from the medium and weighed to measure the water capture and erosion in the formulation. Each batch was tested in triplicate. The swelling ratio (SR) was calculated as the percentage of weight at each time compared to the initial weight of the dried disc.

2.5.6. Drug Release Studies

In vitro drug release was tested to evaluate the drug's dissolution profile in the vaginal environment. Each vaginal disc was placed at the bottom of a borosilicate glass flask with 80 mL of SVF, and the flasks were inserted in a shaking water bath at 37 °C and 15 opm. At predetermined times—0.5, 1, 2, 3, 4, 5, 6, 24 h, and each 24 h from that point—a 5 mL aliquot was removed and filtered, and the medium was replaced with the same amount of SVF in order to maintain a constant volume. Drug concentration was quantified by UV–visible spectroscopy at wavelengths of 260 nm for TFV and 287.5 nm for DPV, using an Evolution 60S spectrophotometer (Thermo scientific, Waltham, MA, USA). Each batch was tested in triplicate. Batches containing DPV were also evaluated using modified SVF containing 5% SDS [10]. This test evaluates the pattern of DPV release from the vaginal discs when solubility is not limited and compares it with the pattern in SVF.

To evaluate if there were statistical differences among batches, the experimental data were compared by the similarity factor (f_2). This is a model-independent index that can be calculated by means of Equation (2); n is the number of samples, R_j the drug release percentage for the reference batch, T_j the drug release percentage for the test, and W_j a weight factor.

$$f_2 = 50 \times log\left\{\left[1 + \left(\frac{1}{n}\right)\sum_{j=1}^{n} W_j|R_j - T_j|\right]^{-0.5} \times 100\right\} \quad (2)$$

2.5.7. Mucoadhesion Time and Force

The adhesion of the vaginal discs to the vaginal mucosa was evaluated with two different methodologies. Mucoadhesion time was determined with a method previously described for the evaluation of vaginal tablets [7]. A portion of freshly excised veal vaginal mucosa obtained from a local slaughterhouse was fixed to a stainless-steel plate with cyanoacrylate adhesive. The vaginal disc was placed over the mucosa, and a force of 500 g was applied for 30 s. The system was placed in a 250 mL beaker, immersed in SVF, and inserted in a shaking water bath (37 °C, 15 opm). The mucoadhesion time was visually observed and established as the time at which the disc detached from the mucosa or completely dissolved in the SFV. This test was performed in duplicate for each batch.

Mucoadhesion force was determined using a Texture Analyser equipped with a 5 kg load cell and a cylindrical probe with a diameter of 1 cm. A sample of vaginal mucosa was fixed on a Petri dish with cyanoacrylate adhesive, and the Petri dish was fixed to the base of the Texture Analyser with double-sided tape. A portion of the vaginal disc was fixed to the probe with double-sided tape and was placed at a height of 40 cm above the mucosa. The probe descended at a speed of 1 mm/s and pressed the mucosa with a force of 500 g for 30 s (the same conditions established in the mucoadhesion time test). After this time, the probe was raised at a speed of 0.1 mm/s, and the force required to detach the disc from the mucosa was recorded [17]. This test was performed in triplicate for each batch. The mucoadhesion force was established as the maximum force required to detach the disc, and the work of mucoadhesion was the area obtained when representing the force required to detach the discs against the time. The results were compared by means of a paired t-test, with $\alpha = 0.05$.

3. Results and Discussion

3.1. Gel Characterisation

3.1.1. Viscosity

Gel viscosity is a basic character that indicates how the structure is affected by the addition of different components. As can be seen in Figure 2, the gel prepared as a reference (H batch) yields a viscosity value close to 4000 mPa·s, which is consistent with the manufacturer's data since we used Methocel K4M (the viscosity value of a 2% gel at 25 °C should be 4000 mPa·s) [30]. This value is similar

to that obtained by other authors when measuring HPMC gels [31]. The high viscosity of this gel suggests it is a good potential candidate to ensure the sustained release of drugs from the freeze-dried gel, as it is able to hydrate quickly and subsequently block any additional uptake of liquids (such as vaginal fluid, which will be required to dissolve the drug so it can be released once the formulation is applied) [32].

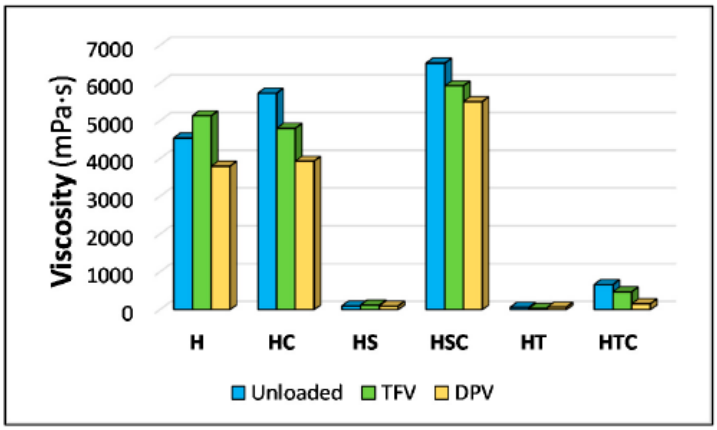

Figure 2. Viscosity of each batch of gels.

It can clearly be seen how the viscosity drops sharply to below 100 mPa·s when a surfactant is added. This effect, which is attributed to the decrease in tension between polymer chains, has been observed for these same surfactants and for many others [33–35]. In these batches, the micelles in the surfactants act as a spatial impediment and prevent the free organization of the HPMC polymer chains and their subsequent gelation. In addition, the surfactants are able to reduce the surface tension of water, which is the main component of the gels, and this parameter has a great influence on the viscosity of the gel. The incorporation of 2HPβCD into the HPMC gel increases the viscosity, as expected due to the viscous nature of this component [36–38]. However, more striking results are seen when cyclodextrins are combined with surfactants; in the combination of 2HPβCD with polysorbate (HTC batch), a rather low viscosity value is obtained, although higher than for the HT batch, indicating that the character of the surfactant, which reduces the viscosity of the gel, predominates over the presence of cyclodextrins. In this batch, the polysorbate cannot interact with the 2HPβCD molecules because its molecules are too large to penetrate the cyclodextrin cavity, so in the absence of a polysorbate/cyclodextrin interaction, the P60 micelles are maintained and prevent the organisation of the HPMC chains, as observed in the HT batch. In contrast, higher viscosity values are obtained in the combination with SDS (HSC batch) than with either of the two components separately, which is noteworthy since SDS, as a surfactant, greatly reduces viscosity. This value for the measure of viscosity points to a possible interaction of SDS with 2HPβCD, in agreement with previous results in the literature [39,40], and indicating that the surfactant may have entered the cyclodextrin cavity. This would reduce the production of SLS micelles, prevent their exposure to interact with the polymer matrix, and eliminate their effect on the gel viscosity. The organisation of HPMC chains is therefore not modified by the presence of the surfactant, which allows its gelation. The incorporation of the drugs also seems to have an influence on the viscosity of the gels. Thus, TFV-loaded gels have a slightly lower viscosity than unloaded gels—except for reference batches. DPV-loaded gels have an even lower viscosity. This may be related to the interposition of the drug among the HPMC chains, which results in difficult interaction among these chains and prevents the polymer from forming the gel structure as occurred in the unloaded batches.

3.1.2. Penetration and Detachment Work

The mechanical properties of the gels were also characterised through an experiment performed on the Texture Analyser, which can be very valuable for observing how the texture profile of the

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gels changes according to their composition [41]. The penetration work (which is a measure of the consistency or strength of the gel) and the detachment work (which is a measure of the adhesiveness of the gel) were quantified.

The results observed in the penetration work clearly show that there are no significant differences when the drugs are included in the gels (Figure 3A). This was statistically demonstrated with a paired t-test (Table 2). Differences in the consistency of the gels are minimal (all batches have strength values between 168–178 g·s), but still apparent. Reference gel (H batch) has the lowest consistency, and when cyclodextrin or any surfactant is included, the consistency increases. In addition, when SDS and 2HPβCD are combined, the consistency is even higher than when they are included separately. However, the most unexpected result is observed in the combination of polysorbate and 2HPβCD, because although the surfactant alone (HT batch) has the highest consistency, the combination (batch HTC) decreases the gel strength. The visual aspect of this batch, where a phase separation is observed in the gel when it is left at rest, suggests that there is an incompatibility among the components at this concentration, and subsequently the gel loses its structure.

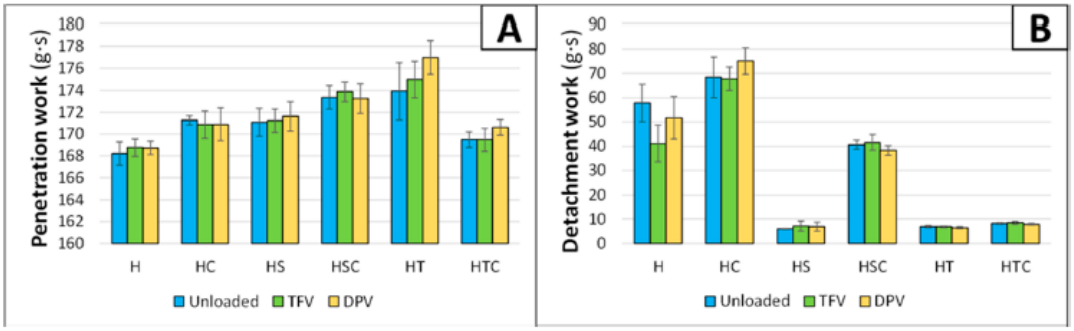

Figure 3. Penetration work (**A**) and detachment work (**B**) for each batch of gels. Mean ± SD are presented ($n = 9$).

Table 2. *p*-Values obtained in the comparison of the group of batches by a paired *t*-test. Significant differences are in bold.

Reference Group	Problem Group	*p* Value ($\alpha = 0.05$)	
		Penetration Work	Detachment Work
Unloaded	TFV-loaded	0.2083	0.4193
Unloaded	DPV-loaded	0.1798	0.8829
TFV-loaded	DPV-loaded	0.2647	0.3584
H	HC	**0.0151**	0.0547
H	HS	**0.0028**	**0.0142**
H	HSC	**0.0016**	0.2025
H	HT	**0.0134**	**0.0124**
H	HTC	0.0640	**0.0138**
HC	HS	0.3661	**0.0013**
HC	HSC	**0.0112**	**0.0120**
HC	HT	**0.0495**	**0.0015**
HC	HTC	0.1234	**0.0016**
HS	HSC	**0.0176**	**0.0011**
HS	HT	**0.0328**	0.9020
HS	HTC	**0.0235**	0.0654
HSC	HT	0.2105	**0.0007**
HSC	HTC	**0.0197**	**0.0007**
HT	HTC	**0.0107**	**0.0052**

The measurements for the detachment work are clearly related to the viscosity of the gels, since the graph (Figure 3B) is similar to the graph for the viscosity values (Figure 2). Again, there are no significant differences between unloaded and drug-loaded batches (Table 2). While the presence of 2HPβCD slightly increases the adhesiveness of the batches, the addition of a surfactant reduces the detachment work. The presence of the surfactant, as commented previously in the viscosity results, decreases the surface tension of the system. The lower surface tension makes it easier for the probe to detach from the gel, and that is the main reason why the inclusion of a surfactant causes a decrease in the detachment work.

In the cyclodextrin and surfactant combinations, as was observed in the viscosity measurements, it is notable that the inclusion of SDS in batches also containing 2HPβCD (HSC batch) maintains the original adhesiveness of the gel (undoubtedly due to the aforementioned interaction). This can be corroborated with the results of the t-test, as it is observed that there is no difference among batches. The combination of polysorbate and 2HPβCD (HTC batch) shows similar detachment work values to the single inclusion of a surfactant, which was also expected due to the phase separation and loss of structure observed in the gel, although minimal differences are apparent in the statistical analysis that makes us think about a minimal increase of adhesiveness due to the presence of the cyclodextrin.

The effect of incorporating cyclodextrins on the texture profile of the gels was expected, since other authors have also described the increase in the hardness, cohesiveness, strength, and adhesiveness of hydrogels [42]. The decline in adhesiveness with the addition of surfactants has also been evaluated and proven with different methods and is attributed to the lower interaction of the polymer with the probe due to the presence of the surfactant [43]. This also explains why the adhesiveness does not vary when SDS and 2HPβCD are combined (HSC batch); the inclusion of SDS in the cyclodextrin renders it unable to prevent contact between HPMC and the probe and maintains the adhesive properties of the polymer.

3.2. Vaginal Disc Characterisation

3.2.1. Apparent Density Calculation

Once the vaginal discs have been manufactured, their appearance and size are analysed to confirm that the manufacturing process is adequate to obtain reproducible discs and to evaluate whether any of the combinations of components undergo alteration during the process. The dimensions of the discs were 50 mm in diameter (determined by the shape of the template used for freeze-drying the gel) and 4.24 ± 0.77 mm in height.

When determining the apparent density of the discs based on their height and weight, it can be seen that the reference batches (H) have the lowest density. The original gels have similar volumes, which will determine the final volume of the disc, although the substance load in each batch varies considerably. In the reference batches, this load is minimal, only 80 mg of HPMC per disc, alone or in combination with 10 mg TFV or 5 mg DPV, so it was expected that these batches had the lowest density values (Figure 4). In contrast, the density is higher in the batches combining cyclodextrins and surfactants due to the greater load of substances in the discs—400 mg of 2HPβCD and/or surfactant. Among batches containing only cyclodextrin or a surfactant, the batches with polysorbate have a much higher density due to the more compact structure obtained in these discs. Finally, in the batches combining 2HPβCD and a surfactant, the discs containing TFV have a higher density as the drug is internalised more effectively in the gel structure. However, when only surfactants are incorporated in the discs, the DPV-loaded discs have a higher density. This is a great advantage for sustained drug release, since the higher density of freeze-dried systems generally implies a smaller average pore size in the structure [44].

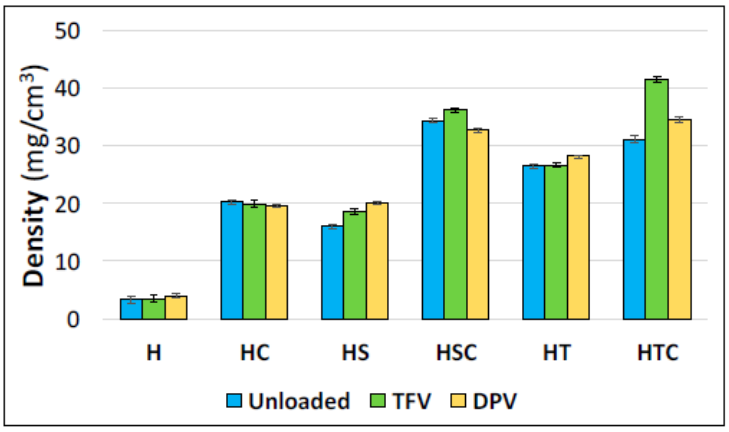

Figure 4. Density values calculated for each batch of discs. Mean ± SD are presented ($n = 5$ for unloaded batches, $n = 8$ for drug-loaded batches).

3.2.2. Porosity Measurement

The size and volume of the pores were subsequently analysed through porosity studies. Figure 5A,C,E show that the total pore volume is not significantly modified regardless of whether the batches are unloaded, TFV-loaded or DPV-loaded. However, there are major differences depending on the components in the discs. H batches have by far the highest total pore volume, and the structure changes significantly when any substance is added, reducing the total free volume. The batches with the next highest pore volume contain only SDS, followed by batches containing only cyclodextrins. The batches that combine these two substances have a slightly smaller total volume, which may be explained by the internalization of the SDS in the structure of the cyclodextrins. The batches that contain only polysorbate have hardly any pores, which again agrees with the denser appearance observed previously. The combination of polysorbates and cyclodextrins produces a structure with a greater volume of pores, suggesting that this surfactant, unlike SDS, is unable to internalize in cyclodextrins and cannot therefore occupy the free volume that 2HPβCD leave in the structure of the discs.

Figure 5B,D,F show the pore size distribution. The batches containing HPMC mostly have pores of around 100 µm, consistent with the sizes observed previously when analysing HPMC gels [7]. The addition of any substance other than drugs significantly changes this distribution. A very curious behaviour can be seen in batches containing cyclodextrins or a combination of SDS with 2HPβCD; although the most predominant pores in the structure of these discs originally have a size of nearly 100 µm (Figure 5B), the addition of TFV (Figure 5D) or DPV (Figure 5F) modifies the gel structure to produce discs with pores of about 50 µm. This confirms the ability of cyclodextrins to modify the structural properties of gels and control the porosity of the materials in which they are included and also to form inclusion complexes with the drugs when they are in an aqueous solution [45]. Finally, it should be noted again that the resulting structure has very low porosity when polysorbate is added.

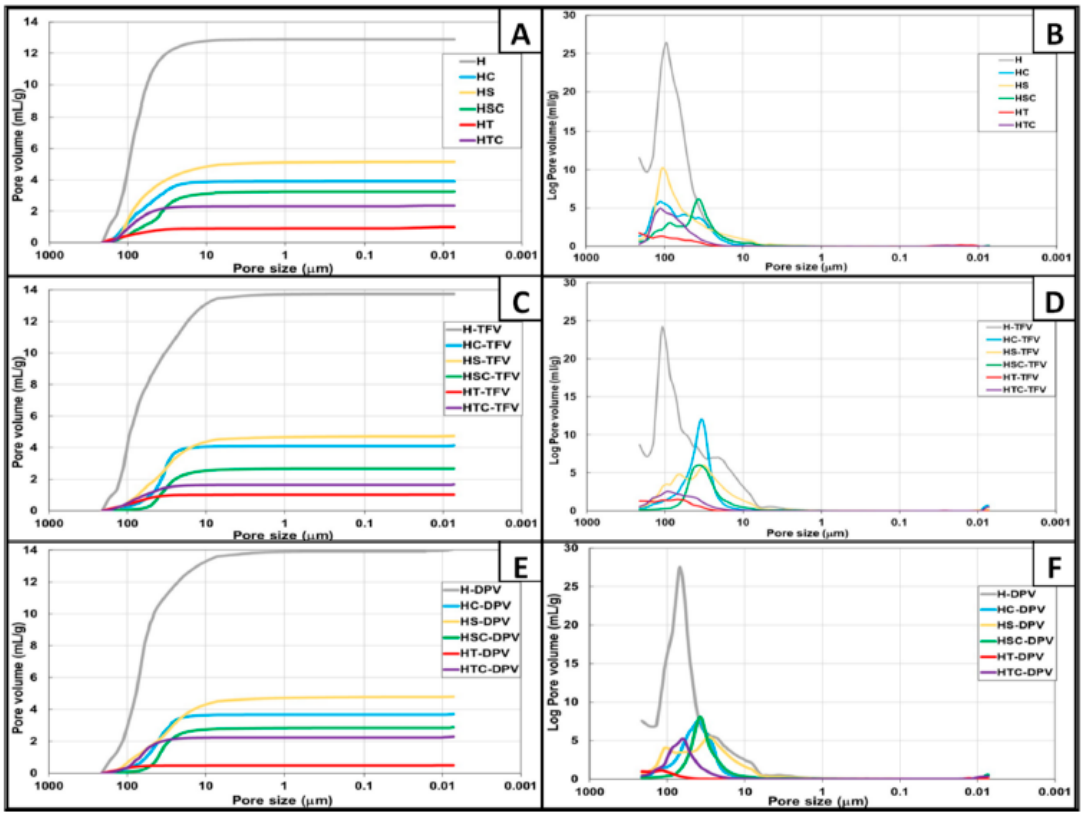

Figure 5. Pore size distributions of vaginal discs, presented as the cumulative volume of unloaded (**A**), TFV (**C**), and DPV (**E**) batches and the logarithm of the differential pore volume of unloaded (**B**), TFV (**D**), and DPV (**F**) batches.

3.2.3. Scanning Electron Microscopy (SEM) Micrographs

The inner structure of the discs was observed through SEM (Figure 6). As can be seen in these micrographs, H batches with or without drugs become very structured freeze-dried gels with large pores (around 100μm), which matches the medium pore size observed in Hg porosimetry and is also similar to the structure of other HPMC-based systems [28,46]. The incorporation of 2HPβCD maintains the porous structure although with smaller pores. This phenomenon, i.e., the decrease in pore size with the inclusion of cyclodextrins in the polymer structure, is also observed in the combination with other polymers such as chitosan [47]. When HC batches are loaded with TFV (a water-soluble drug) the pores appear somewhat smaller, and the inclusion of DPV (a poorly water-soluble drug) makes the pores even smaller. These changes in pore size when drugs are included were also noted in porosimetry studies. Batches with only SDS form completely unstructured freeze-dried gels with a fragile appearance. This was also seen in the photographs in Figure 1, but this fragility required confirmation through the analysis of mechanical properties described below. In contrast, batches containing the other surfactant (polysorbate) have a compact appearance with no visible pores (also agreeing with the results for Hg porosimetry and the greater density of these systems).

The analysis of the structure of discs combining cyclodextrins and surfactants reveals some interesting aspects of the formulations. In the combination of 2HPβCD and SDS, it is notable that although no defined structure is observed in the absence of drug, a structure with micropores is formed when TFV or DPV is incorporated. The presence of the drug therefore helps stabilize the interaction between SDS and 2HPβCD. The discs obtained when mixing polysorbate and 2HPβCD in HPMC gels have a completely heterogeneous structure, again revealing the incompatibility of the components.

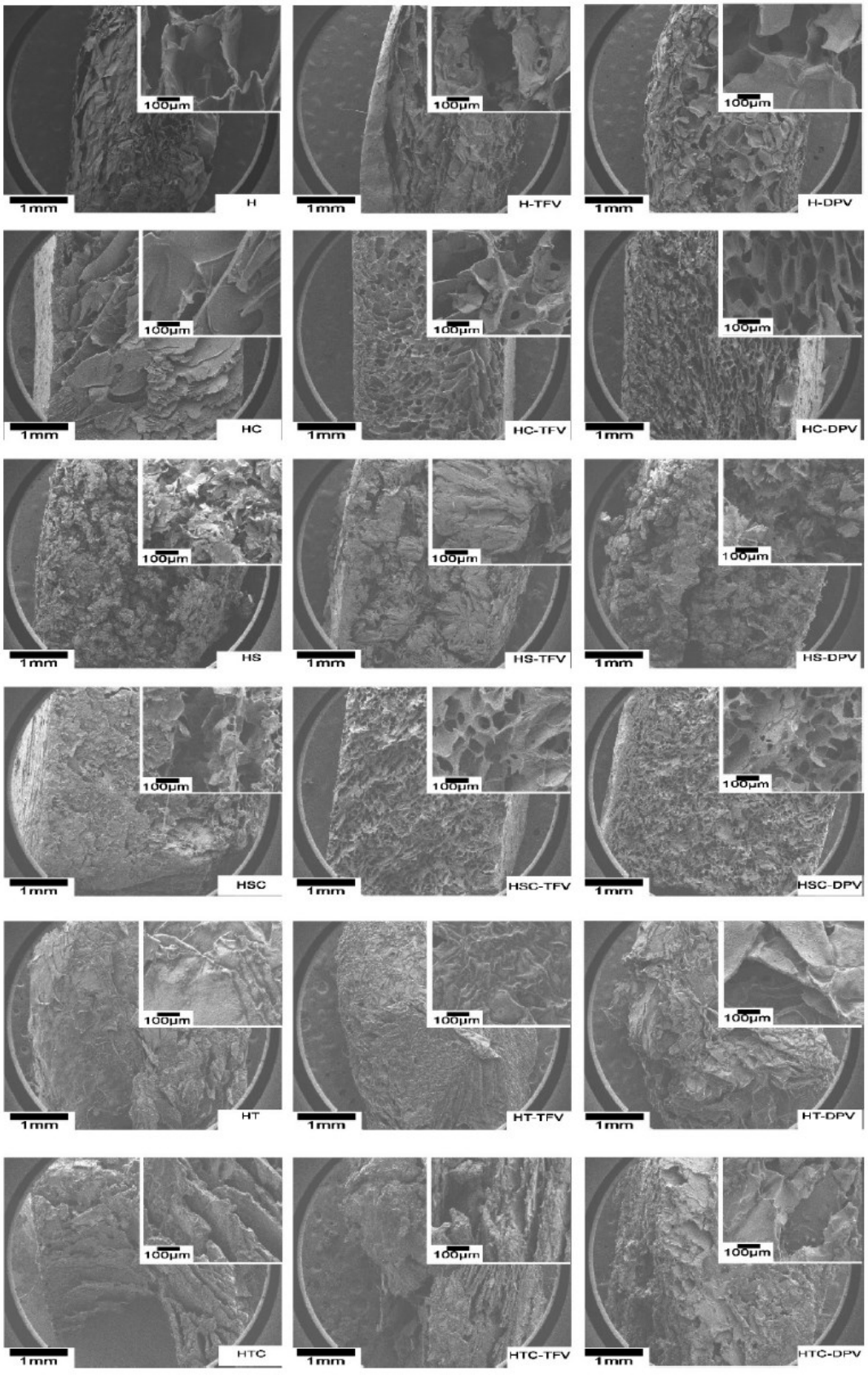

Figure 6. Scanning electron micrographs of vaginal discs.

3.2.4. Mechanical Properties: Deformation and Resistance to Fracture

The mechanical properties of the discs were also evaluated to determine whether they are capable of withstanding the stress involved during their administration. An analysis of the deformability of the discs reveals that the inclusion of a surfactant, either SDS or polysorbate, decreases the deformability of the formulations (Figure 7), whereas the addition of cyclodextrins substantially modifies the structure of the discs, making them much more robust and resistant to deformation, since more than double the force is required to deform them than if cyclodextrins are not included. The combination of 2HPβCD and SDS offers high resistance, although slightly lower than in batches containing only cyclodextrins, while the combination with polysorbate gives the discs a similar resistance to batches that include only surfactants, once again suggesting that 2HPβCD is not properly incorporated into the disc structure. In general, the results confirm the premise that the smaller the size of the pores that form the structure of freeze-dried systems, the greater their deformability [17].

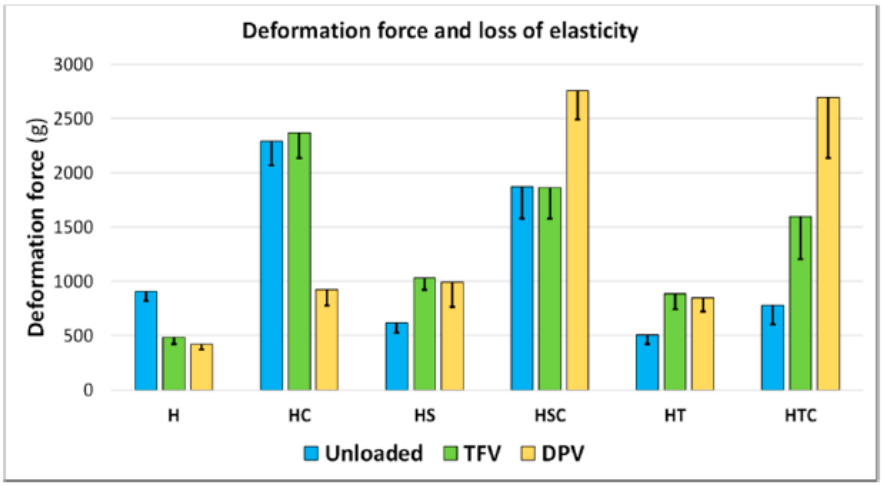

Figure 7. Mean values of the deformation force required to deform vaginal discs a distance of 1 mm (n = 4). Inner bars represent the loss of elasticity after 10 deformation cycles.

When analysing the influence of drugs, the findings are more disparate. In the reference batches (with only HPMC), the addition of drugs makes the structure more deformable, possibly because the active principle is not uniformly incorporated. In batches with 2HPβCD, this same phenomenon is observed only with the addition of DPV, so TFV can be homogeneously incorporated into the structure when cyclodextrins are present. The addition of surfactants appears to facilitate the incorporation of the drugs, since the complex surfactant-drug forms discs with a more robust structure. The same occurs when surfactants are combined with 2HPβCD, although in these cases, batches that include DPV are particularly notable (HSC-DPV and HTC-DPV) for withstanding the greatest deformation force.

The loss of deformability of the discs is minimal in the batches incorporating only HPMC and increases with the addition of cyclodextrins or surfactants separately; it is even greater in the combination with both solubilizing agents. This is consistent with other studies that evaluate the deformability of freeze-dried systems based on cellulose derivatives, since adding more substances to the systems increases their loss of elasticity [48].

The fracture resistance of solid vaginal formulations, whether tablets or films, is always evaluated to ensure that they are suitable for vaginal administration [49–52]. An analysis was also conducted on the force required to rupture the discs and the distance they are deformed before breaking; the results are shown in Figure 8. It should first be clarified that less force is required to break the discs than to deform them in the previous test, as the probes used to evaluate these parameters are different: a large probe for flat surfaces is used to compress the disc in the deformation test, while the rupture test

consists of a puncture made with a probe with a circular head with a small diameter. Since the force is applied to a smaller surface, logically less force is required.

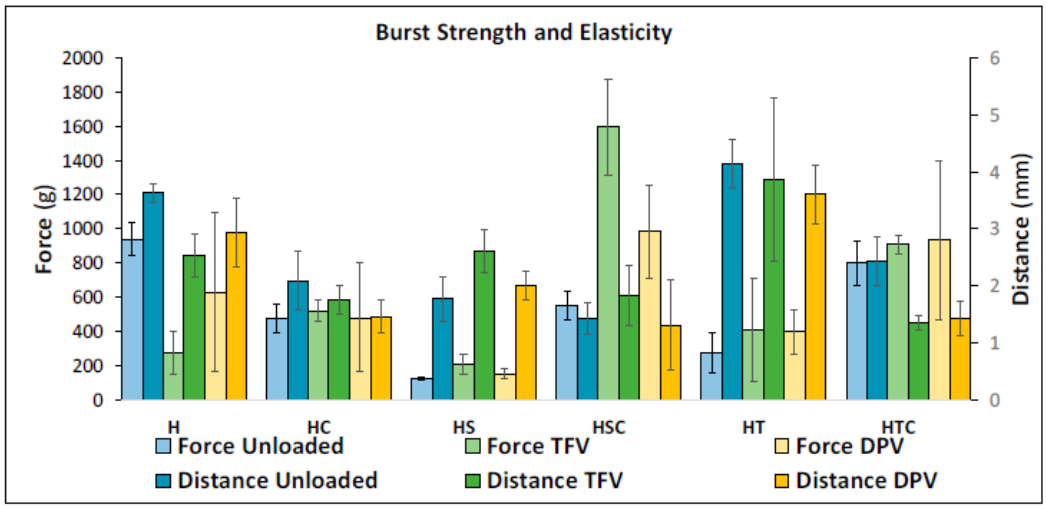

Figure 8. Burst strength and distance to burst of vaginal discs. Mean ± SD are presented ($n = 4$).

The most notable finding in the fracture resistance test is that the presence of surfactants significantly modifies the mechanical properties of the discs, but in a quite different way. While the addition of SDS makes the systems more fragile so they withstand the least force before breaking (confirming our hypothesis after observing the SEM micrographs that these discs had an unstructured appearance that could cause the fragility of the formulation), the discs become much more elastic when polysorbate is incorporated and are able to deform a greater distance with the application of minimal force. This can also be explained by the results of the previous tests, since the denser and thinner structure of these discs confers better mechanical properties. Other authors report that freeze-dried gels become harder the larger the pore diameter [53], and as can be seen in Figure 8, this is also true in our case, since the reference disc (H batch) withstands the most force before breaking and also has the highest pore diameter.

Another point worth mentioning based on the results is that there are no significant differences between the systems when drugs are incorporated, whatever their nature. However, there is one exception to this statement, and these are the batches that combine 2HPβCD and SDS. As seen in the porosimetry studies (and confirmed in the SEM micrographs), the addition of drugs causes these systems to form a more robust structure with smaller pores. This characteristic also appears to affect the mechanical properties of the discs, since they are capable of deforming a significantly greater distance before breaking, which makes these formulations very suitable for vaginal administration.

3.2.5. Swelling Behaviour

A study was done to determine the behaviour of the discs in the presence of SVF once placed inside the vagina. As this is a freeze-dried system, the discs can be assumed to rapidly capture water after immersion to reconstitute the original gel. As can be seen in Figure 9, this occurs in all batches except those combining polysorbate and cyclodextrins, confirming the incompatibility between these components at the ratios evaluated; they do not form a homogeneous gel, and the freeze-dried system is subsequently unable to rehydrate so the gel can recover at a faster rate than the erosion of the disc. All the other batches swell quickly and reach their maximum swelling ratio at 30 min, except for batches containing only HPMC which have a much more marked swelling and can capture about six times more water than the others. Their maximum swelling ratio is reached 1 h after immersion, which agrees with the results of other authors when evaluating freeze-dried systems based on HPMC [54].

This greater swelling ability is clearly related to the results of the previous test for these batches; since these discs have the highest total pore volume and the largest average pore size, they can absorb a greater amount of SVF. These batches also take longer to completely dissolve, as can be seen in Figure 9.

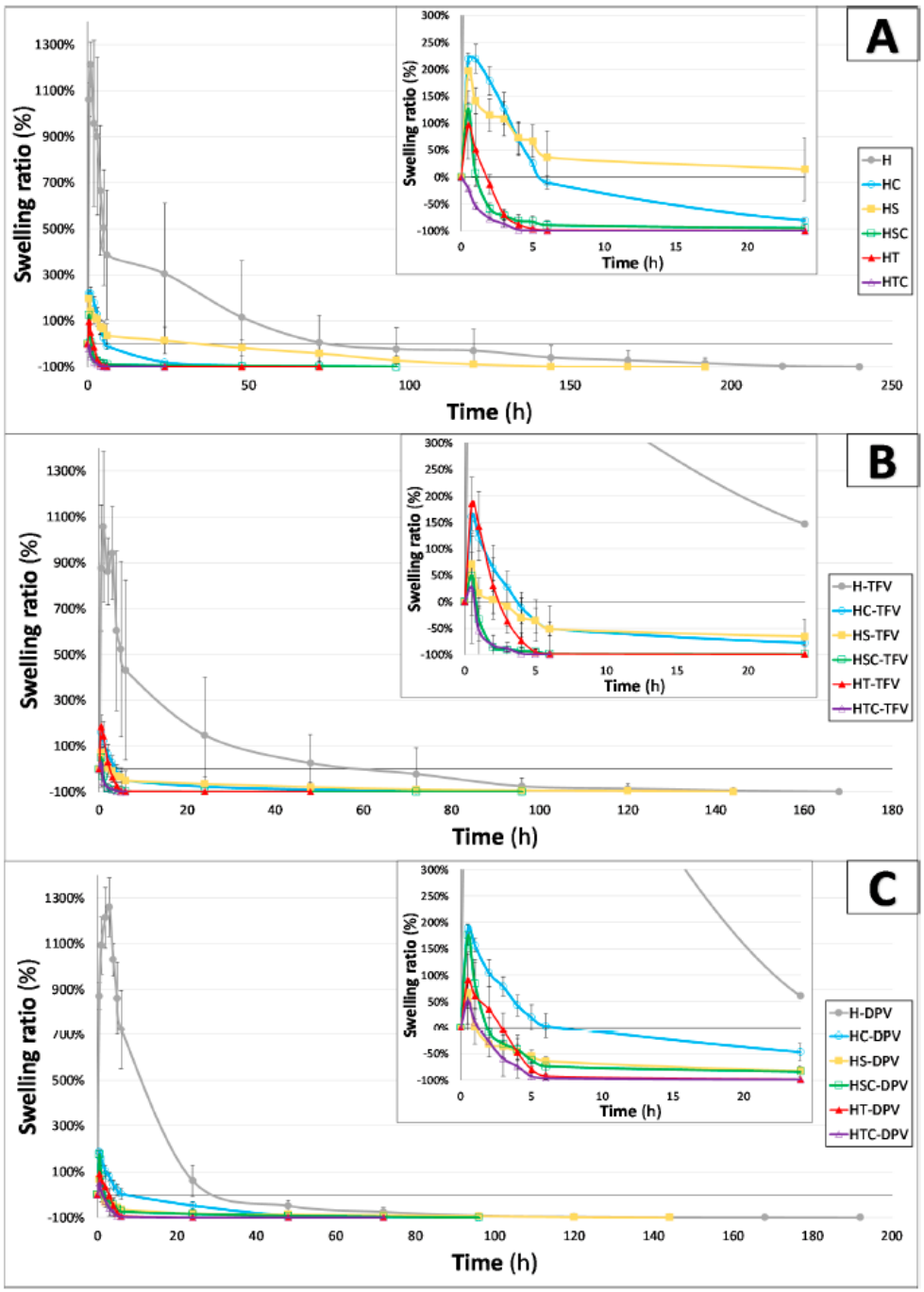

Figure 9. Swelling ratio profile of unloaded (**A**), TFV-loaded (**B**), and DPV-loaded (C) vaginal discs immersed in simulated vaginal fluid. The values obtained up to 24 h of trial are expanded inside each picture. Mean ± SD are presented (n = 3).

Similarly, the moderate swelling of the other batches (HC, HS, HSC, and HT) is directly related to the decrease in total pore volume as seen in the porosimetry studies. There are no significant differences

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between these batches in terms of swelling profiles and almost all are largely dissolved after 24 h. The decrease in the swelling ratio of hydrogels in the presence of cyclodextrins was also expected, not only because of the inability of the 2HPβCD to swell, but also due to the modification of the gel structure [55]. Finally, there are again no significant differences in terms of swelling profile with the addition of drugs to the discs. Only the batches containing only SDS show any difference; when a drug is included in the system, it captures a lesser amount of SVF and is eroded faster. This can be explained by that fact that the complex formed by the drug and the surfactant behaves differently in the presence of the medium than when the SDS is mixed directly in the structure of the HPMC gel.

3.2.6. Drug Release Studies

Undoubtedly the most important feature to evaluate in drug-loaded vaginal discs is the drug release behaviour when the drugs are administered. This was simulated with an in vitro trial, where the discs were immersed in SVF under sink conditions.

When analysing the results of the drug release studies for TFV-loaded vaginal discs, the most interesting finding is the control over drug release achieved when 2HPβCD is incorporated in the HPMC structure (Figure 10). These batches are able to release the drug in a sustained manner for 7 h, a notable improvement on the 4 h release time of the reference batch (H-TFV). This proves that the inclusion of cyclodextrins not only contributes to increasing the solubility of drugs but also controls the release of the active principle [56]. In this batch, the control of TFV release is due to the lower swelling ability of the formulation, as seen previously in the swelling test. The smaller pores in its structure hinder the entrance of the medium in the system, thus delaying the TFV diffusion.

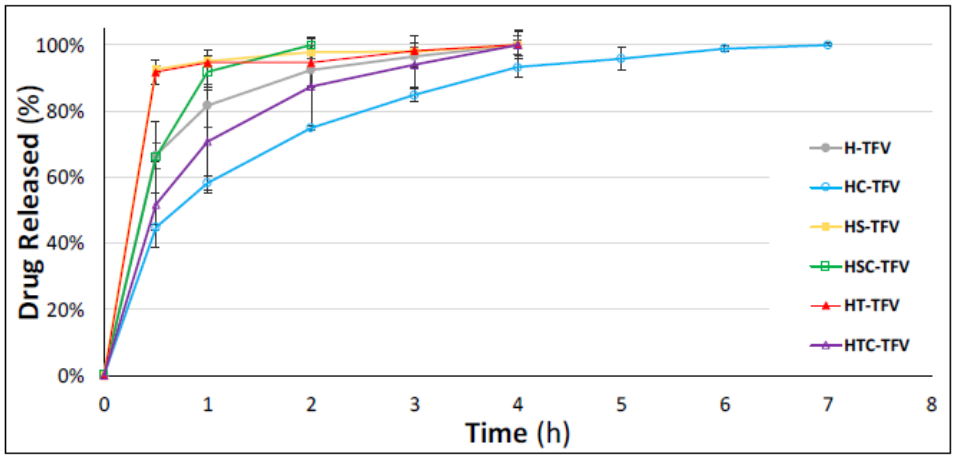

Figure 10. Drug release profiles of TFV-loaded vaginal discs in simulated vaginal fluid. Mean ± SD are presented (n = 3).

In contrast, the addition of a surfactant (SDS or polysorbate) markedly accelerates the dissolution of the drug, and both HS-TFV and HT-TFV batches release around 90% of the drug in 30 min. There are no significant differences in drug release as a function of the surfactant included in the formulation (Table 3). This occurs for two reasons: the higher solubility of TFV in SVF in the presence of the micelles of both surfactants, and the faster erosion of the discs when these substances are included. Nevertheless, in the f2 comparison, it is possible to observe variations among the batches combining these surfactants with the cyclodextrin. This is also appreciable in the drug release graphic; while the combination of SDS with 2HPβCD is capable of achieving the fastest complete release of the drug (in 2 h), the cyclodextrin and polysorbate combination has a similar release profile to the reference batch. However, the HTC-TFV batch shows the highest standard deviation values, undoubtedly due to the erratic release caused by the heterogenicity of this system.

Table 3. Similarity factor (f_2) values obtained from the comparison of batches. Significant differences are in bold.

Reference	Problem	f_2 Value		
		TFV-loaded SVF	DPV-loaded SVF + SLS	DPV-loaded SVF
H	HC	**37.0**	65.1	72.5
H	HS	**36.7**	**27.5**	**15.3**
H	HSC	61.0	**27.8**	**14.0**
H	HT	**37.4**	**28.2**	**19.3**
H	HTC	51.0	**34.2**	**30.1**
HC	HS	**23.6**	**24.7**	**15.7**
HC	HSC	**31.9**	**24.9**	**14.5**
HC	HT	**24.0**	**25.1**	**20.6**
HC	HTC	51.0	**30.4**	**32.5**
HS	HSC	**36.3**	**43.3**	**43.3**
HS	HT	96.6	56.1	**33.1**
HS	HTC	**26.9**	**33.6**	**20.7**
HSC	HT	**37.0**	60.0	**36.9**
HSC	HTC	**41.5**	**46.9**	**22.8**
HT	HTC	**27.4**	**44.8**	**38.3**

Since DPV is very poorly soluble in SVF, making it difficult to achieve sink conditions, alternative media must be used to evaluate the drug release profiles of DPV-loaded vaginal formulations [57]. Most authors use organic solvents to evaluate the release of DPV [58], but while this may be a good strategy for hydrophobic formulations (such as vaginal rings), it can cause problems when evaluating swellable systems due to the different swelling behaviour in this medium compared to vaginal fluid. However, a good alternative is simulated vaginal fluid modified with the addition of surfactants to increase DPV solubility and achieve sink conditions [59,60]. We have therefore used the medium previously reported by Cazorla-Luna et al., which includes 5% SDS in SVF, to evaluate the DPV release profile from the discs under sink conditions [10].

As can be seen in Figure 11, batches containing SDS, alone or in combination with 2HPβCD, show the fastest DPV release, with all the drug released in just 2–3 h. Batches including polysorbate (and again their combination with cyclodextrin) also have a very fast release, with all the DPV released in 4–6 h. The reference batch and the disc containing only 2HPβCD (H-DPV and HC-DPV) are therefore capable of moderate drug release, which is sustained for 24 h in both cases. In addition, it is appreciable that there is no statistical difference in the release profile when only the cyclodextrin is included (Table 3). This also confirms that the inclusion of a surfactant notably modifies the structure of the discs as well as improves DPV release, since the micelles of the surfactant favour the dissolution of poorly water-soluble drugs. As can be seen in the statistical analysis, batches HS and HT are similar, although again there are differences when the surfactants are combined with 2HPβCD (HSC and HTC batches). This can be explained due to the different ability to interact with the cyclodextrin of each surfactant agent.

However, as the main aim of including DPV in vaginal discs was to achieve a formulation capable of helping DPV to fast-release in the presence of vaginal fluid, the batches were also evaluated in SVF to observe how they would release the drug in the vaginal environment.

This drug release study reveals no significant differences when cyclodextrin is included (HC-DPV) compared to the reference batch (H-DPV) (Table 3). This confirms that DPV is unable to form inclusion complexes with 2HPβCD, so the solubility is not improved: none of these batches are able to release more than 2.5 mg of DPV after 48 h (Figure 12).

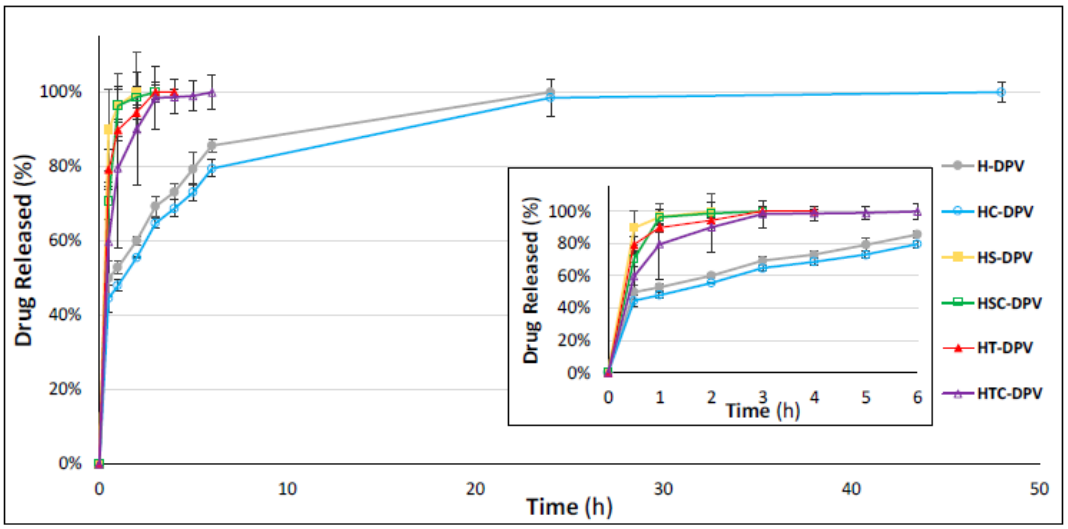

Figure 11. Drug release profiles of DPV-loaded vaginal discs in modified simulated vaginal fluid (including 5% SDS). The values obtained up to 6 h of trial are expanded inside the picture. Mean ± SD are presented ($n = 3$).

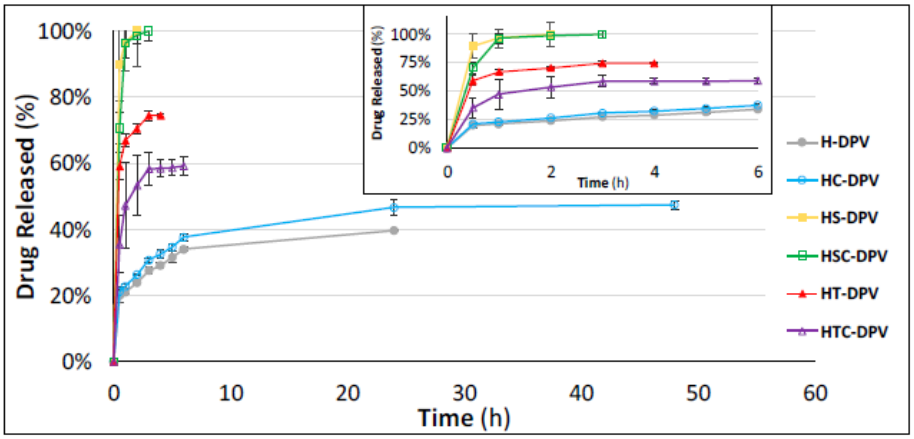

Figure 12. Drug release profiles of DPV-loaded vaginal discs in simulated vaginal fluid. The values obtained up to 6 h of trial are expanded inside the picture. Mean ± SD are presented ($n = 3$).

Nevertheless, each of the other batches is significantly different, as is observed in the f_2 comparison. The addition of polysorbate increases the total amount of drug released, but is still unable to completely dissolve the 5 mg of drug in the formulation. It is striking that although the total amount of surfactant is the same for HT-DPV and HTC-DPV batches, the final drug dissolved is lower when cyclodextrins are included. The aforementioned incompatibility between P60 and 2HPβCD causes the polysorbate micelles not to exhibit their solubilising power, which is why the drug release profile from HT-DPV is greater than that from HTC-DPV.

In contrast, the inclusion of SDS achieves the complete dissolution of the DPV in the disc, so it is potentially a good strategy to achieve a DPV-loaded formulation capable of fast release once administered vaginally. The combination of SDS with 2HPβCD shows the same drug release profile, but it is worth highlighting the usefulness of including cyclodextrins; due to the vaginal cytotoxicity of free SDS (it has already be proven that high concentrations of this surfactant can cause vaginal ulcers), it is imperative to evaluate the toxicity of any formulation that includes this substance before testing in clinical trials [61]. Since it has been demonstrated that SDS is able to form a complex with 2HPβCD, this complex is also probably less cytotoxic than when SDS is in a free state, as occurs in the

combination of cyclodextrins with other cytotoxic substances [62], so the inclusion of cyclodextrins in this system may be valuable, not in terms of drug release, but from a safety point of view. Obviously, the amount of SDS included in the discs must also be adjusted and reduced to the minimum required for drug solubility.

3.2.7. Mucoadhesion Time and Force

Finally, the adhesive properties of the vaginal discs were also evaluated in order to demonstrate that the discs can be retained in the vagina after their administration, since their possible expulsion would lead to the subsequent failure of the formulation to protect against infection.

Mucoadhesion time was evaluated, and the results are shown in Figure 13. It should first be mentioned that only batches H, HS, and HSC (as well as their drug-loaded equivalent batches) are capable of remaining adhered until their complete dissolution. Batches containing only 2HPβCD, only polysorbate, or a combination of both, detached before they were completely dissolved. The reference batches (containing only HPMC) have by far the longest mucoadhesion times, undoubtedly due to their higher swelling ratio, which is directly related to the mucoadhesive properties [51,63].

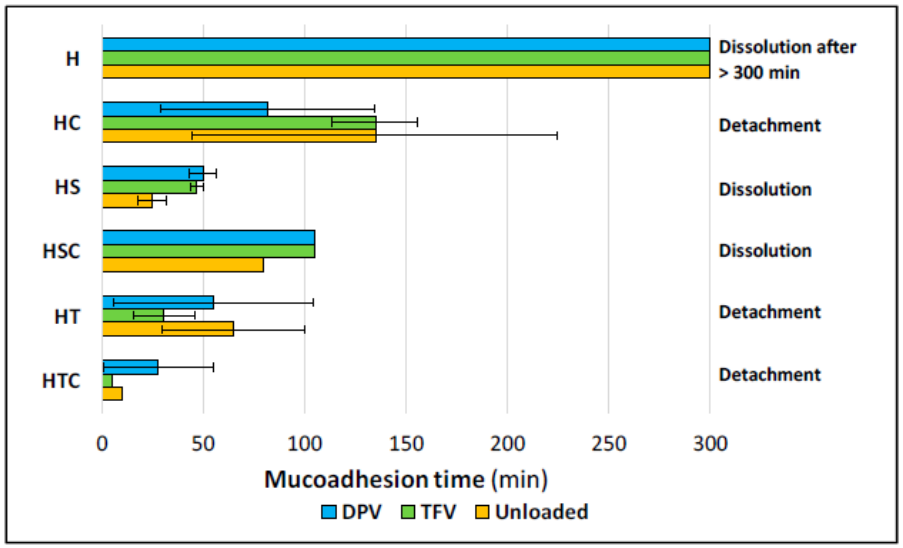

Figure 13. Mucoadhesion time until the detachment of the vaginal discs. At right is detailed if mucoadhesion time is limited by the detachment or complete dissolution of the disc. Mean ± SD are presented ($n = 2$).

The fact that the addition of SDS causes the system to remain adhered until its complete dissolution is because it is an anionic surfactant that enables the discs to bond to the mucosa due to electrostatic charges and with hydrogen bonds formed by HPMC [64,65]. In contrast, the inclusion of 2HPβCD scarcely modifies the disc structure so it also retains most of the adhesive potential of HPMC; these batches have the second longest mucoadhesion time after the reference batches. However, possibly the most suitable batches in terms of mucoadhesion time combine SDS and cyclodextrins, since they still have a good mucoadhesion time (due to the presence of 2HPβCD) and can also remain attached to the vaginal mucosa until complete dissolution (due to the presence of SDS). It should also be noted that there are no significant differences in mucoadhesion time when the drugs are included in the formulations.

However, when evaluating mucoadhesive force, greater adhesiveness is observed in batches containing only SDS (Figure 14). This is because of the bonding by electrostatic charges, which are stronger than hydrogen bonds [7]. This is statistically demonstrated by means of paired t-test analysis, where it is observed that HS batches differ from batches with cyclodextrin, polysorbate, and their

combination (Table 4). Again, the combination of SDS and 2HPβCD shows an adequate mucoadhesion force, which is even greater than the force obtained in reference batches when TFV or DPV is included in the vaginal discs. No significant differences are observed when unloaded batches are compared with batches containing any drug, so it can be concluded that the addition of drugs to the discs barely modifies their mucoadhesive properties (Table 4).

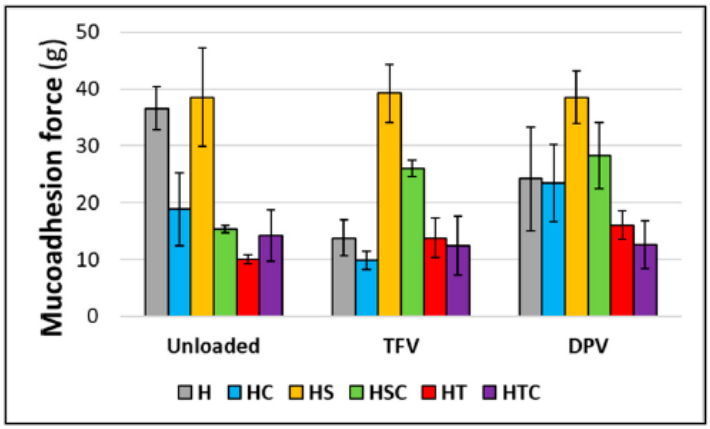

Figure 14. Mucoadhesion force required to detach the vaginal discs. Mean ± SD are presented (n = 3).

Table 4. p-Values obtained in the comparison of the group of batches by paired t-test. Significant differences are in bold.

Reference Group	Problem Group	p-Value
Unloaded	TFV-loaded	0.5441
Unloaded	DPV-loaded	0.6698
TFV-loaded	DPV-loaded	0.1095
H	HC	0.2851
H	HS	0.1769
H	HSC	0.8837
H	HT	0.2778
H	HTC	0.1916
HC	HS	**0.0364**
HC	HSC	0.4117
HC	HT	0.4179
HC	HTC	0.3869
HS	HSC	0.0577
HS	HT	**0.0047**
HS	HTC	**0.0008**
HSC	HT	**0.0492**
HSC	HTC	0.1563
HT	HTC	0.9514

Therefore, while HS batches are notable for their excellent mucoadhesion force, HC batches stand out for their adhered residence time. Thus, HSC batches are the most adequate in terms of mucoadhesion, as they have an acceptable adhesion force and are able to remain adhered until their complete erosion.

In view of the results, it can be confirmed that vaginal discs have great potential for vaginal administration of microbicides. Although the current trend is to search for dosage forms that allow a sustained release of drugs in order to stagger the administration interval, another alternative for better patient compliance is to develop coitally-dependent formulations that are administered by women just before intercourse and offer immediate protection.

TFV-loaded discs clearly achieve this objective, as with the inclusion of surfactants they are able to release practically all the drug in only half an hour. The formulation also proved to be useful for the rapid release of a lipophilic drug, DPV, despite its poor solubility in the vaginal environment.

Another advantage of vaginal discs compared to other vaginal dosage forms comes from their unlikely interference with intercourse. It is clear that solid dosage forms—especially vaginal rings—can be detected by men during intercourse and can cause discomfort [66]. Moreover, vaginal gels usually cause leakage and messiness. Nevertheless, vaginal discs would capture the volume of fluid already present in the vagina to form a gel, so the formulation would not result in an additional volume in the vaginal environment. This behaviour is similar to that already observed in vaginal films [67]. In addition, the volume the discs capture—as observed in the swelling test—would be lower than 2 mL, so the volume can be considered acceptable. Finally, it should also be mentioned that the stress during intercourse will not only affect the efficacy of the formulation but could even accelerate the dissolution of the system. The inclusion of cyclodextrins in these formulations performs numerous functions: 2HPβCD modulates the release of TFV, significantly improves the mechanical properties of the formulations (without which they would be too fragile to administer), and particularly increases the adhesiveness of the formulation to the vaginal mucosa, which is essential for it to be retained in the vaginal environment until all of the drug is released. If we compare the adhesiveness force of the developed formulations to vaginal films developed for the administration of antiretroviral drugs, we can appreciate that the force required to detach both formulations from the vaginal mucosa is really similar [68]. Cyclodextrins are also considered to play a fundamental role in counteracting the cytotoxicity of SDS.

Future studies are needed to adjust the dose of drug administered, as the discs have been shown to hold and release large drug doses, 10 mg of TFV and 5 mg of DPV, and consequently, the amount of surfactant and cyclodextrins necessary to reduce the possible toxicity to safe levels.

4. Conclusions

Vaginal discs based on HPMC freeze-dried gels are a pharmaceutical form suitable for the fast release of vaginally administered drugs.

The inclusion of SDS in HPMC gels allows DPV (a poorly water-soluble drug) to be incorporated and dissolved in the vaginal medium at the same rate as TFV (a hydrophilic drug).

The combination of SDS and 2HPβCD modifies the microstructure of the HPMC gel and produces freeze-dried systems with smaller pores, forming a final structure with a lower pore volume, which not only allows the inclusion and release of over 90% of the drugs, both TFV and DPV, in just half an hour, but also creates a robust structure that gives the discs greater mechanical strength and better mucoadhesive properties. This fast release would guarantee rapid protection after placing it in the vagina.

The administration of vaginal discs based on HPMC freeze-dried gels immediately prior to sexual intercourse could offer women a comfortable tool for protection against the sexual acquisition of HIV. The comfortable administration, and especially the on-demand use, may be translated into better adherence to the use of this microbicide by women, which has been the main problem observed to date with vaginal microbicides.

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DISCUSIÓN INTEGRADORA

La revisión del estado en que se encontraba el desarrollo de microbicidas vaginales para la prevención de la transmisión sexual del virus de inmunodeficiencia humana (VIH), abordada en el capítulo I de la presente tesis, constituye el punto de partida para la investigación llevada a cabo.

Como se ha podido ver en dicho capítulo, el desarrollo histórico de las formulaciones microbicidas comenzó con el uso de agentes que pudiesen inactivar el virus mediante mecanismos físicos (por ejemplo, con sustancias surfactantes) o mediante una unión química con el mismo (como se evaluó a través del uso de anticuerpos monoclonales o polianiones). También fue explorada la posibilidad de crear una barrera física que impidiera su contacto con las células del epitelio vaginal, o de exponer al virus a un medio más adverso que dificultara su estabilidad, gracias a agentes acidificantes. Sin embargo, se corroboró que los microbicidas desarrollados mediante este tipo de estrategias no fueron capaces de demostrar su utilidad en ensayos clínicos.

En consecuencia, el uso de microbicidas cargados con fármacos antirretrovirales se antoja la estrategia más favorable para el desarrollo de una formulación que pudiese proteger a la mujer de la transmisión sexual del VIH. Este tipo de productos son los más desarrollados actualmente en dicho campo de estudio, y alguno de ellos ha logrado mostrar resultados esperanzadores en los ensayos clínicos. De entre las numerosas sustancias activas evaluadas, decidimos seleccionar para la presente tesis las dos que habían demostrado mayor eficacia hasta la fecha. Se trata del tenofovir (TFV) y de la dapivirina (DPV). La selección de dichas sustancias implica además un reto, pues su solubilidad en medios acuosos difiere notablemente – mientras que el TFV es un fármaco hidrosoluble, la DPV se caracteriza por ser prácticamente insoluble en agua –. Esto implicará que deban estudiarse distintas estrategias, recursos tecnológicos e incluso formulaciones para lograr desarrollar un microbicida adecuado a las características del fármaco incorporado.

En cuanto a las formas farmacéuticas empleadas hasta la fecha, como se ha detallado en el primer capítulo, existe una amplia variedad. Los geles vaginales están presentes en la gran mayoría de estudios dentro del campo de los microbicidas vaginales, pues históricamente han sido considerados como la formulación más aceptada por las mujeres para su administración por vía vaginal. Sin embargo, los

comprimidos, los *films* y los anillos vaginales son formas de dosificación alternativas a los geles que también han sido objeto de estudio para el fin que se contempla. No se deben dejar de mencionar los recursos tecnológicos más novedosos que han sido aplicados para el desarrollo de microbicidas vaginales. Así, existen referencias al uso de electrohilados o de organismos modificados genéticamente que, si bien aún se encuentran en una temprana fase de desarrollo, podrían ser de gran utilidad en el futuro debido a las ventajas que les proveen de gran potencial para su aplicación terapéutica. Por supuesto, la nanotecnología también ha irrumpido notablemente en este campo de estudio. Prueba de ello son las cada vez más frecuentes formulaciones que recurren a la misma para otorgar características mejoradas a las formas de dosificación en que se incorporan.

Para la selección de la forma de dosificación en que se basarían los primeros estudios desarrollados en esta tesis (capítulos II a V) se consideraron diversos factores. Dado que las potenciales usuarias de dicha formulación serían principalmente mujeres en países de ingresos medios y bajos – pues como se ha detallado en el primer capítulo, la incidencia de nuevas infecciones es más prevalente en el África subsahariana –, es necesario utilizar una forma farmacéutica de bajo coste, así como materias primas que no incrementen significativamente el precio del producto. Los comprimidos aparecen como la opción ideal para tal fin. Se trata de la forma farmacéutica más ampliamente utilizada a nivel mundial por la industria farmacéutica, con un coste de producción bastante económico y una tecnología de fabricación ampliamente conocida e implementada. Además, presentan una gran estabilidad frente a condiciones ambientales adversas – lo que supone otra ventaja, dadas las elevadas condiciones de temperatura y humedad que se pueden llegar a registrar en estas regiones – y son fáciles de transportar, manejar y administrar.

En cuanto a la estrategia de liberación y posología de elección para el microbicida a desarrollar, se toma una decisión en base a los estudios recogidos en este primer capítulo. Como ya se ha desarrollado anteriormente, inicialmente se buscaba el uso de formas farmacéuticas de liberación inmediata. Sin embargo, a raíz de los resultados obtenidos en el estudio *CAPRISA 004* – donde se pudo observar una gran variación en la eficacia protectora como resultado de la frecuencia de administración del gel vaginal de

TFV – se optó por potenciar el desarrollo de formulaciones de liberación sostenida. De este modo se lograría mantener la eficacia protectora durante mayores periodos de tiempo, reduciendo la frecuencia de administración del microbicida y en consecuencia dando lugar a una posología más cómoda para las usuarias, que podría conllevar una mejor adherencia al tratamiento profiláctico.

En base a lo expuesto anteriormente, el trabajo de laboratorio para esta tesis comenzaría con el diseño y desarrollo de comprimidos de administración vaginal para la liberación sostenida de TFV. Se optó por comenzar por este principio activo ya que es el que menor dificultad conlleva para su posterior evaluación (dado su carácter hidrofílico). En cuanto al objetivo propuesto, se consideraría aceptable una liberación sostenida del fármaco durante al menos siete días, pudiendo así sugerir una aplicación semanal del microbicida desarrollado, frente al uso diario que requerían la mayoría de microbicidas en estudio hasta la fecha.

En el capítulo II se establece la base para el desarrollo de estos comprimidos de liberación sostenida de TFV. Para ello, se evalúan comprimidos fabricados con cuatro polímeros diferentes: hidroxipropilmetil celulosa (HPMC), quitosano, goma guar y Eudragit® RS. El objetivo principal de este capítulo es la caracterización de cada uno de estos polímeros, para así evaluar sus propiedades y poder determinar su utilidad en el planteado desarrollo de los comprimidos. Es por ello que se fabricaron comprimidos mediante la compresión de mezclas físicas del fármaco con cada uno de estos polímeros – con una pequeña cantidad de estearato magnésico, que actúa como lubrificante para facilitar el proceso de compresión –.

En el trabajo incluido en este capítulo se evalúa el potencial de estos comprimidos para sostener la liberación de TFV en el medio vaginal. Como se puede ver, tanto HPMC como quitosano presentan buenas características de retención del fármaco, pues estos comprimidos eran capaces de liberar el TFV de manera controlada durante 48 h. Además, para un mejor conocimiento de dicho proceso se realizan estudios de hinchamiento. En estos estudios es posible apreciar el proceso de gelificación y posterior erosión que sufren HPMC, quitosano y goma guar cuando entran en contacto con fluido vaginal simulado, y que varía en cada uno de ellos. Además, el hinchamiento podría tener una gran influencia en la aceptación de la forma

farmacéutica por parte de las usuarias, pues un comprimido que captase una gran cantidad de agua podría llegar a resultar incómodo. Del mismo modo, si se producen fugas del gel formado podría suponer tanto una disminución de la eficacia de la formulación como un rechazo por parte de las usuarias. En este sentido, tanto HPMC como goma guar demostraron tener una elevada capacidad de captación de agua al gelificar, que podrían conllevar los mencionados inconvenientes. Para conocer mejor la estructura del gel formado se obtuvieron los innovadores testigos de hinchamiento; el comprimido era extraído del medio en su momento de máximo hinchamiento, era liofilizado para extraer el agua absorbida sin alterar la estructura, y el esqueleto del gel era analizado mediante estudios de porosimetría y a través de microscopía electrónica de barrido. Por último, pero no menos importante, se realizó una evaluación del tiempo de mucoadhesión de los comprimidos a la mucosa vaginal. Esta característica resulta esencial al querer obtener comprimidos de liberación sostenida, pues debemos garantizar que el microbicida es retenido en la vagina durante el tiempo necesario para que tenga lugar la liberación completa del antirretroviral. En esta ocasión, el quitosano destacó por ser el polímero que peores propiedades mucoadhesivas presentaba de entre los evaluados. Además, se comprobó mediante estudios de citotoxicidad en cultivos celulares que todos los polímeros estudiados eran seguros para su administración a las dosis frecuentemente utilizadas.

Como fruto del trabajo expuesto en este segundo capítulo se pudo obtener una idea más definida de las características que ofrecía cada uno de los polímeros estudiados, pero lamentablemente se llegó a la conclusión de que ninguno de ellos reunía todas las características ideales para la fabricación de comprimidos de liberación sostenida de TFV.

En el capítulo III se estudia el potencial de los cuatro polímeros utilizados en el capítulo anterior para complementar sus propiedades, así como evaluar posibles efectos sinérgicos. Es por ello que se decide fabricar comprimidos en los que se combinen aquellos polímeros con una alta capacidad de hinchamiento (HPMC y goma guar) con los dos polímeros que presentaban una captación más moderada de fluido vaginal (quitosano y Eudragit® RS). Cada una de las posibles combinaciones se evaluó en tres proporciones de mezcla de polímeros distintas. Además, se incluyó un agente

estructural (difosfato cálcico anhidro), y los comprimidos fueron fabricados a través de granulados obtenidos mediante granulación vía húmeda (utilizando polivinilpirrolidona como humectante). De este modo, se busca dar una mayor robustez a los comprimidos desarrollados.

Los estudios llevados a cabo en este capítulo son los mismos que en el capítulo anterior, para así poder comparar mejor los resultados obtenidos. De este modo fue posible observar como la combinación de HPMC y quitosano resultaba de gran potencial para el fin perseguido. Se observó un efecto sinérgico en cuanto al control de la liberación del fármaco, pues mientras que los polímeros por separado habían mostrado 48 h de liberación sostenida, en este caso fue posible obtener 72 h en las que el fármaco era liberado de forma controlada. En cuanto a la mucoadhesión, la presencia de HPMC conseguía contrarrestar la pobre capacidad adhesiva mostrada por los comprimidos de quitosano, consiguiendo que los comprimidos elaborados con las mezclas de polímeros permaneciesen adheridos a la mucosa vaginal por un periodo de 96 h. Este tiempo garantizaría la retención de la formulación hasta que todo el fármaco incluido haya sido liberado. Por último, los resultados más interesantes serían observados en los estudios de hinchamiento. Con la mezcla de polímeros fue posible conseguir un hinchamiento homogéneo de la formulación – el quitosano había mostrado en el capítulo anterior un hinchamiento por capas que causaba la ruptura de los comprimidos –. Además, la presencia de quitosano moderaba el excesivo hinchamiento que había sido observado en los comprimidos basados en HPMC. En consecuencia, con la mezcla de polímeros se conseguían comprimidos que resultarían previsiblemente más cómodos para su administración vaginal, con la consecuente aceptación de las usuarias.

Por lo tanto, de los estudios llevados a cabo en el tercer capítulo se pudo concluir que la mezcla de HPMC y quitosano mostraba un gran potencial para el desarrollo de comprimidos de liberación sostenida de TFV, pues se conseguía aunar en una formulación las ventajas que cada uno de estos polímeros ofrecían al ser evaluados por separado.

Para el capítulo IV de la presente tesis se optó por mantener la mezcla de HPMC y quitosano seleccionada en el capítulo anterior, y realizar modificaciones en la forma

de incluir el principio activo en los comprimidos, para así intentar prolongar el tiempo mediante el cual podrían ofrecer protección frente a la transmisión sexual del VIH.

Se propone un granulado de TFV junto con dos ingredientes con alta hidrofobia, como son el Eudragit® RS y la zeína. Diferentes proporciones de TFV son incorporadas a los gránulos, para así evaluar también el efecto de la proporción TFV/Eudragit® RS o TFV/zeína sobre la liberación del principio activo. La idea es incorporar una barrera más para la difusión del fármaco al exterior. Dado que se han utilizado sustancias insolubles en el medio acuoso, será necesaria la difusión del TFV desde los gránulos para poder acceder al medio vaginal. Estos gránulos son mezclados con un granulado obtenido como en el capítulo anterior, con HPMC, quitosano, difosfato cálcico anhidro y polivinilpirrolidona. La mezcla de granulados es comprimida en las mismas condiciones que en el capítulo tercero. Los estudios previos llevados a cabo con el granulado (difracción de rayos X, espectroscopía infrarroja, termogravimetría y calorimetría diferencial de barrido) demostraron la estabilidad del fármaco durante el proceso de granulación.

En cuanto a las propiedades de los comprimidos resultantes, en primer lugar se observó una menor capacidad de captación de fluido vaginal. Este efecto puede asociarse al hecho de que, al incluir los gránulos, se está incluyendo una carga de materia hidrófoba en el comprimido que dificulta la difusión del fluido a través del mismo. Del mismo modo, el tiempo de retención de la formulación adherida a la mucosa vaginal se ve incrementado por la presencia de los gránulos. Desde las 96 h observadas en la formulación desarrollada en el capítulo anterior, pasan hasta 120-240 h en función de la proporción y naturaleza de los gránulos incorporados. Esto se puede asociar nuevamente a la menor captación de agua lograda como consecuencia de la incorporación de los gránulos, pues varios estudios encuentran una relación entre un hinchamiento más moderado y una mejor mucoadhesión.

Respecto a la liberación de TFV desde los comprimidos, destaca la formulación en la que el principio activo fue granulado junto con Eudragit® RS, en una proporción fármaco/polímero 1:2. Estos comprimidos lograban liberar el TFV incluido en los gránulos durante 144h, permaneciendo adheridos a la mucosa durante 150 h – por lo que garantizan su retención durante el tiempo necesario para la liberación –. Es

indudable que suponen un gran avance respecto a los comprimidos desarrollados en el capítulo anterior para lograr el fin de desarrollar un microbicida de liberación prolongada de TFV.

Para el capítulo V de esta tesis se incide en el proceso de granulación del fármaco de forma previa a su incorporación en los comprimidos basados en HPMC y quitosano. En esta ocasión se recurre a las sustancias comercializadas como Gelucire®. Concretamente son utilizados Gelucire® 43/01, Gelucire® 39/01 o una mezcla de ambos para, mediante fusión de los mismos y posterior granulación, obtener nuevamente granulados mixtos de TFV con estas sustancias altamente hidrófobas. La estrategia, por tanto, es la misma que ha sido desarrollada en el capítulo anterior. Sin embargo, esta vez se busca que la temperatura corporal juegue un papel en la liberación del fármaco. Así, hasta que el Gelucire® no consiga fundir como consecuencia de la temperatura, el fármaco se verá retenido en los gránulos. Además, en este proceso se evalúan nuevas sustancias, así como un nuevo proceso de obtención del granulado.

Precisamente este proceso, que implica la fusión en caliente del Gelucire® antes de incorporar el TFV, requiere llevar a cabo un estudio en profundidad de la estabilidad del TFV tras el proceso de granulación. Para ello, además de volver a aplicar las técnicas utilizadas en el capítulo anterior, se evalúan los granulados mediante termodifracción de rayos X (para ver cómo se modifica la cristalinidad del TFV a medida que se incrementa la temperatura, y si este proceso es reversible). También se utiliza la microscopía de platina caliente para observar de forma visual los cambios en el principio activo detectados mediante las otras técnicas utilizadas. Como consecuencia de este estudio se puede concluir que el TFV mantiene sus propiedades tras el proceso de granulación llevado a cabo, lo que es esencial para garantizar su eficacia terapéutica.

En cuanto a la evaluación de los comprimidos, nuevamente es observado que la adición de los gránulos reduce el hinchamiento de la formulación e incrementa el tiempo de mucoadhesión. Para comentar los resultados obtenidos en la liberación merece la pena mencionar que, al utilizarse Gelucire® con puntos de fusión de 39 °C, 43 °C y una mezcla de ambos, era de esperar que los mejores resultados se obtuviesen con el Gelucire® 39/01, por presentar una temperatura de fusión más próxima a la temperatura corporal. Sin embargo, aunque en las horas iniciales todas las

formulaciones presentaban un comportamiento similar, a partir de las 6 h se observó que el control sobre la liberación del TFV era mayor cuanto mayor era el punto de fusión del Gelucire® empleado. La explicación para este fenómeno es que cuando el Gelucire® funde completamente el fármaco es capaz de salir al exterior sin apenas dificultad. En cambio, cuando los gránulos son preparados con Gelucire® 43/01 éste no funde a la temperatura corporal pero sí se reblandece en la presencia del fluido vaginal caliente, haciendo posible la lenta difusión del fármaco a través de este Gelucire® reblandecido.

Los comprimidos de HPMC y quitosano que incluían gránulos de TFV/Gelucire® 43/01, en proporción 1:2, fueron capaces de liberar el fármaco de manera sostenida durante 216 h (9 días), permaneciendo adheridos a la mucosa vaginal durante todo ese tiempo. Esto supone que el objetivo de desarrollar comprimidos de liberación sostenida de TFV que permitieran su aplicación semanal por vía vaginal habría sido logrado con esta formulación. Evidentemente, puesto que todos los resultados de la presente tesis son desarrollados en estudios *in vitro* o *ex vivo*, será necesario llevar a cabo en el futuro estudios que permitan evaluar si los datos obtenidos son extrapolables en estudios *in vivo*, así como para garantizar la seguridad y la eficacia de la formulación en modelos animales.

Para dar continuidad a la tesis, la siguiente etapa consistiría en el desarrollo de una forma farmacéutica alternativa a los comprimidos vaginales. En este sentido, se decidió optar por el desarrollo de *films* vaginales, ya que consideramos que se trata de una formulación que está atrayendo un gran interés en los últimos años. Además, dadas sus características, se podría decir que aúnan las ventajas de formulaciones sólidas y semisólidas (son más cómodos y fáciles de autoadministrar para las usuarias que otras formulaciones más voluminosas, no requieren de un aplicador, evitan las fugas de producto tras su aplicación, son estables frente a condiciones adversas y fáciles de transportar).

En el capítulo VI se realiza una revisión bibliográfica sobre el desarrollo de *films* de administración vaginal. En esta ocasión la idea principal del capítulo no es conocer los microbicidas vaginales desarrollados en esta forma farmacéutica, pues eso ya se estudió en el primer capítulo, sino profundizar en el desarrollo y caracterización de la forma farmacéutica con la que se va a trabajar.

A lo largo de este sexto capítulo se detallan los diferentes tipos de *films* que podemos encontrar en la actualidad basándonos en su solubilidad en el fluido vaginal, en su estructura o en el mecanismo de liberación de fármacos. Además, se han recopilado los distintos ingredientes formadores de *films* que han sido utilizados hasta la fecha en la fabricación de *films* vaginales, y son clasificados en función de su naturaleza. Como cada una de estas sustancias requiere de unos requisitos específicos para la obtención de *films*, también han sido recopilados los distintos parámetros que deben ser controlados durante el proceso de fabricación. Del mismo modo, los plastificantes utilizados en *films* vaginales hasta la fecha también son detallados en este capítulo, clasificándolos en función de su naturaleza. La primera conclusión que se puede sacar con la información recopilada es que, en función de la naturaleza del polímero empleado para la fabricación de los *films*, será necesario incluir plastificantes con unas características determinadas para asegurar su compatibilidad.

También se ha profundizado en las diferentes técnicas que han sido aplicadas hasta la fecha para la fabricación de *films* vaginales, comentando los parámetros del proceso que tendrán influencia en las propiedades de la formulación final. En este sentido, el método de evaporación del solvente destaca por ser el más utilizado en los artículos de investigación que podemos encontrar en la bibliografía. Es por ello que se opta por esta técnica para la fabricación de *films* vaginales en el marco de la presente tesis, debido a que se trata de un método bastante bien conocido – como muestra la gran información disponible – y de económica aplicación a escala de laboratorio. Sin embargo, en este sexto capítulo también han sido comentadas técnicas alternativas, tales como el electrohilado, el electroesprayado, el moldeo por extrusión o compresión, o incluso la impresión 3D.

A continuación, en este mismo sexto capítulo son recogidas las técnicas de caracterización utilizadas hasta la fecha por los investigadores en el campo de los *films* de administración vaginal. Este trabajo de búsqueda bibliográfica resulta esencial, pues al no existir una normativa específica en cuanto a los ensayos de caracterización que debe superar esta forma farmacéutica para su comercialización, existen una gran cantidad de metodologías que precisaban de ser comparadas y comentadas para determinar su utilidad y aplicabilidad. Las distintas metodologías utilizadas para

caracterizar la uniformidad de los *films*, su hidrofilia o hidrofobia, sus propiedades mecánicas o su estructura son recogidas en esta sección. La aplicación de las técnicas de imagen y las técnicas térmicas al análisis de los *films* también resulta de gran utilidad, pues permite conocer mejor su estructura. Por supuesto, también es necesario realizar ensayos que permitan explorar las propiedades adhesivas o el control sobre la liberación de fármacos desde esta forma farmacéutica. Por último, como ocurre con el resto de formas de dosificación, son requeridos estudios de estabilidad, así como estudios de seguridad y eficacia tanto *in vitro* como *in vivo*. Es por ello que se ha recogido la forma de adaptar estas técnicas al estudio de *films* vaginales.

Para terminar con este capítulo, las distintas aplicaciones hasta la fecha de los *films* vaginales también han sido recopiladas, pues es imprescindible conocer los últimos avances en el campo de estudio antes de comenzar la investigación en el mismo. En este sentido, destacan claramente los *films* vaginales destinados al tratamiento y prevención de enfermedades de transmisión sexual, tales como el VIH, la candidiasis vulvovaginal, o la vaginosis bacteriana. También se han utilizado en otras aplicaciones, como el desarrollo de *films* vaginales anticonceptivos o para el tratamiento de la disfunción sexual femenina.

El trabajo incluido en este capítulo podría resultar en una guía de gran utilidad tanto para investigadores como para el desarrollo a nivel industrial de *films* vaginales, pues resume y actualiza la tendencia actual en la fabricación de esta forma farmacéutica. Para el desarrollo de la presente tesis, obviamente también resulta de gran utilidad. La búsqueda bibliográfica ha servido para conocer mejor la versatilidad de técnicas y materias primas que pueden ser utilizadas para la fabricación de los *films*, así como las potenciales ventajas de esta forma farmacéutica sobre otras. Del mismo modo se ha podido comprobar que, aunque inicialmente los *films* vaginales únicamente fueron considerados como un recurso para la rápida administración de fármacos, en los últimos años están surgiendo alternativas que valoran la posibilidad de utilizar esta forma farmacéutica para la liberación controlada.

En el capítulo VII, una vez recopilada y analizada la información recogida en el capítulo anterior, se decide comenzar con el desarrollo de *films* vaginales para la liberación sostenida de TFV. En este sentido, la estrategia a seguir es la misma que la

desarrollada entre los capítulos II y V de esta tesis, pero en esta ocasión se buscará su aplicación a una forma farmacéutica alternativa, como son los *films*.

Para el desarrollo de estos *films* se evalúa el potencial de un polímero ya conocido y utilizado en el desarrollo de los comprimidos vaginales, como es la HPMC. Por otro lado, y en contraposición a la alta hidrofilia y capacidad de gelificación del derivado celulósico, se utilizará también la zeína. Esta proteína derivada del maíz, ya utilizada en el capítulo IV para la obtención de granulados, posee una naturaleza anfifílica en la que predominan los restos hidrófobos.

Para conocer las características de cada una de estas sustancias se fabricaron *films* con dos concentraciones diferentes. Además, se evalúa la influencia que tienen sobre las propiedades de estos *films* la incorporación de agentes plastificantes, así como la cantidad de plastificante en el *film*. En este sentido, puesto que la HPMC es claramente hidrófila, se incorporan dos plastificantes polares pero con muy diferente peso molecular – glicerol y polietilenglicol 400 –. Por otro lado, para plastificar la zeína han sido utilizados el ácido oleico y el trietilcitrato, sustancias con naturaleza anfifílica e hidrófoba, respectivamente. Las propiedades de los *films* desarrolladas son evaluadas a través de estudios de flexibilidad, resistencia, elasticidad, análisis térmico, bioadhesión, hinchamiento, erosión y liberación del fármaco en fluido vaginal. En este sentido, mientras que los *films* de HPMC destacan por sus propiedades mecánicas, los *films* basados en zeína se caracterizan por otorgar mejores propiedades para la liberación sostenida de TFV – hasta 96 h, en contraposición a las 24 h ofrecidas por los *films* de HPMC –. Todos los *films* mostraron excelentes propiedades bioadhesivas. También ha sido comprobado, mediante estudios de citotoxicidad en líneas celulares MT-2, THP-1 y HEC-1-A, que ninguno de los componentes es tóxico a dosis moderadas.

En consecuencia con los resultados obtenidos se decidió que una combinación de HPMC y zeína, en las proporciones y con el plastificante adecuado, podría dar lugar a un *film* de administración vaginal que aunase las ventajas de ambos polímeros. Los resultados obtenidos son incluso mejores de lo esperado, pues la combinación de HPMC y zeína en proporción 1:5, junto con incorporación de un 40% de polietilenglicol 400 como plastificante, daba lugar a unos *films* con adecuadas propiedades. No solo la presencia del HPMC otorgaba buenas propiedades mecánicas a la formulación, sino que

además la estructura resultante de la combinación de estas sustancias permitía la liberación sostenida de TFV durante 120 h. Es por ello que esta formulación podría ser evaluada en futuros estudios *in vivo*, para así determinar si su aplicación clínica podría ser de utilidad para la prevención de la transmisión sexual del VIH.

Para el siguiente capítulo de esta tesis se decide modificar la estrategia a seguir para conseguir mejorar la adherencia al tratamiento profiláctico por parte de las usuarias. Así, se deja atrás el desarrollo de formulaciones de liberación sostenida y se inicia el desarrollo de *films* inteligentes para la liberación pH-dependiente de TFV. La idea para estas formulaciones es conseguir un *film* que, si bien lograra retener el fármaco en la presencia de fluido vaginal, cuando se produce la eyaculación – y el pH vaginal se ve notablemente alcalinizado –, se acelere la liberación del principio activo. Este tipo de formulaciones podrían ser de gran utilidad para facilitar la posología, pues permitirían ser aplicadas en cualquier momento previo a las relaciones sexuales y tan solo liberarían el antirretroviral en el momento en que se produce la eyaculación, que es cuando existe mayor riesgo de contagio.

En el capítulo VIII, se dan los primeros pasos en la búsqueda de un *film* vaginal para la liberación pH-dependiente de TFV. Puesto que en el capítulo anterior ya se había logrado optimizar un *film* vaginal que permitiera sostener el fármaco en un medio ácido, como el fluido vaginal, se decide partir de la combinación de HPMC y zeína en proporción 1:5 que tan buenos resultados había dado. Sin embargo, ninguna de estas dos sustancias se caracteriza por tener un comportamiento pH-dependiente, por lo que no serían adecuadas para conseguir esa modificación en la velocidad de liberación tras la alcalinización del medio. Es por ello que, en este octavo capítulo, evaluamos si la inclusión de agentes plastificantes de distinta naturaleza – acidez, hidrosolubilidad, etc. – podría modificar la liberación del TFV en función del pH.

En una primera etapa se evalúa la influencia de la incorporación, por separado, de cuatro agentes plastificantes – polietilenglicol 400, glicerol, ácido láctico y ácido oleico –. De los resultados obtenidos se puede concluir que el ácido oleico es el único que otorga al *film* la capacidad de liberar más rápidamente el fármaco cuando se encuentra en contacto con un medio alcalino. Sin embargo, el polietilenglicol 400 es con diferencia el polímero que mejor sostiene la liberación del principio activo en el fluido

vaginal – como ya se pudo comprobar en el capítulo anterior –. Además, las propiedades mecánicas de los *films* son notablemente mejores al incorporar ácido láctico o polietilenglicol, mientras que las propiedades mucoadhesivas mejoran al incorporar ácido oleico o ácido láctico.

En la siguiente etapa de desarrollo de estos *films* se evalúa la combinación en proporciones iguales de los agentes plastificantes con carácter ácido – ácido láctico y ácido oleico – con los plastificantes neutros – glicerol y polietilenglicol 400 –. Los resultados revelan que sólo los lotes que incorporan ácido oleico muestran diferencias significativas entre el perfil de liberación a distintos pH. De entre ellos, la combinación de ácido oleico y polietilenglicol 400 resulta la más adecuada. En este lote, las propiedades mecánicas de los *films* son mejoradas gracias a la incorporación del polietilenglicol 400, pero la excelente adhesividad que se observó en los *films* de ácido oleico se ve empeorada.

Para la tercera fase de desarrollo de estos *films* se fabrican formulaciones en las que se combinan ácido oleico y polietilenglicol, pero en diferentes proporciones de plastificantes. Los resultados revelan claramente que, cuanto más ácido oleico incluyen los *films*, más se acelera la liberación al alcalinizar el medio. La proporción ácido oleico/polietilenglicol 400 7:1 es seleccionada para preparar los siguientes lotes de *films*. En estos lotes, manteniendo siempre esta proporción, se varía la proporción de plastificante incorporado respecto a la cantidad de HPMC y zeína en el *film*. Como conclusión, podemos observar que los *films* que combinan ácido oleico/polietilenglicol 400 en proporción 7:1, con un 80% de plastificante respecto al peso de los agentes formadores de la matriz del *film*, son capaces de duplicar la velocidad de liberación de TFV cuando el medio es alcalinizado. Además, dada la gran cantidad de ácido oleico incorporado, los *films* presentan excelentes propiedades mucoadhesivas. En cuanto a las propiedades mecánicas de los *films*, la presencia de polietilenglicol 400 hace que la formulación final tenga mejor flexibilidad y resistencia.

En consecuencia, en este octavo capítulo se ha podido comprobar que el desarrollo de *films* de liberación pH-dependiente de fármacos no solo es posible a través de las propiedades de los polímeros, sino que las características de los plastificantes

incluidos en la formulación también producen cambios significativos en las propiedades del *film*.

A continuación, el capítulo IX de esta tesis continúa con la estrategia de desarrollar *films* vaginales de liberación pH-dependiente de TFV. Sin embargo, la investigación sufre un reinicio en cuanto a los materiales utilizados para obtener tal fin. En este capítulo será evaluado el potencial de distintos polímeros comercializados bajo la marca Eudragit®. Se trata de derivados del ácido metacrílico frecuentemente utilizados en el recubrimiento de formulaciones sólidas – por lo que se les presuponen buenas propiedades formadoras de *films* –. Además, algunos de ellos son habitualmente empleados en la obtención de cubiertas funcionales, que modifican su solubilidad o permeabilidad en función del pH del medio.

Es por ello que en este noveno capítulo realizamos un cribado para evaluar el potencial de cuatro derivados del ácido metacrílico – Eudragit® RS, Eudragit® RL, Eudragit® S y Eudragit® L – para la fabricación de *films* vaginales de liberación pH-dependiente de TFV.

El trabajo se inicia con la preparación de *films* basados en cada uno de estos cuatro polímeros, mediante la técnica de evaporación del disolvente, evaluando a su vez cuatro solventes distintos – metanol, etanol, acetona o isopropanol –. Como conclusión se pudo observar que las propiedades mecánicas varían notablemente en función del disolvente utilizado. Se decide seleccionar el isopropanol como el disolvente más adecuado, pues los *films* basados en los cuatro tipos de Eudragit® presentan una flexibilidad aceptable al ser fabricados con este disolvente.

En la siguiente fase, en la que todos los lotes son fabricados tras su disolución en isopropanol, se evalúa la incorporación de seis plastificantes diferentes sobre las propiedades de cada uno de los polímeros. Se emplean dos polioles – glicerol y polietilenglicol 400 –, dos ácidos – láctico y oleico – y dos derivados del ácido cítrico – trietilcitrato y tributilcitrato –. De cada pareja de plastificantes se selecciona el más adecuado en función de las propiedades mecánicas del *film* resultante, y en esta ocasión sí que se observan diferencias significativas entre los distintos polímeros. Así, mientras que los *films* fabricados con Eudragit® RS y Eudragit® RL presentaban mejores

propiedades al ser plastificados con glicerol, ácido oleico y tributilcitrato, los *films* basados en Eudragit® S y Eudragit® L resultaban más compatibles con polietilenglicol 400, ácido láctico y trietilcitrato.

A continuación, el objetivo es determinar la concentración ideal de cada plastificante para cada tipo de Eudragit®. Es por ello que cada polímero es plastificado con los agentes plastificantes seleccionados en la fase anterior, pero en cuatro concentraciones diferentes – 20%, 40%, 60% y 80% –. Nuevamente se evalúan las propiedades mecánicas de los *films* resultantes, consiguiendo así determinar la concentración de plastificante ideal para cada polímero. Las posibles interacciones entre polímero y plastificante son observadas mediante espectroscopía infrarroja, que también sirve para comprobar la ausencia del disolvente – isopropanol – en los *films* preparados.

Los *films* seleccionados en la fase anterior son evaluados en términos de liberación de fármaco cuando se encuentran inmersos en fluido vaginal simulado. Para cada uno de los polímeros, se seleccionan aquellos *films* que incluyen el plastificante que les permite sostener la liberación del TFV durante el mayor tiempo posible en este medio ácido. Así, se seleccionan finalmente cuatro lotes: Eudragit® S plastificado con un 20% de ácido oleico, Eudragit® RL plastificado con un 40% de glicerol, Eudragit® S plastificado con un 40% de trietilcitrato y Eudragit® L plastificado con un 80% de trietilcitrato. Estos cuatro lotes fueron evaluados también en una mezcla de fluido vaginal simulado y fluido seminal simulado, para así comprobar si alguno de ellos es útil para la aceleración de la liberación del principio activo tras la eyaculación. Tanto los *films* basados en Eudragit® S como especialmente aquellos fabricados con Eudragit® L demostraron un gran potencial para el desarrollo de *films* vaginales de liberación pH dependiente de TFV, pues en ambos casos se conseguía que la liberación del fármaco tuviese lugar en menos de 6 h tras la alcalinización del medio, mientras que en un medio ácido el TFV era retenido por más de 200 h. Además, estudios de permeabilidad *in vitro* demostraron que el tenofovir liberado desde estas formulaciones presentaba una menor permeabilidad celular – lo que reduciría su distribución sistémica –, mientras que era capaz de acumularse asociado a las monocapas celulares en niveles similares al tenofovir libre, garantizando su concentración en el lugar de acción.

En consecuencia, queda probado que estos dos polímeros podrían tener una enorme aplicación para el desarrollo de *films* vaginales de liberación pH-dependiente de TFV. En el futuro se podría evaluar su combinación con otros agentes formadores de *films*, ya sea en *films* mixtos o en *films* multicapa, para así mejorar aún más las propiedades que pueden ofrecer.

Para concluir la presente tesis se decide explorar una tercera estrategia para mejorar la posología de las formulaciones microbicidas: los microbicidas de acción inmediata. La idea en esta ocasión es buscar un uso a demanda del producto, que podría ser aplicado únicamente en el momento inmediatamente previo a las relaciones sexuales y lograr una protección prácticamente instantánea.

En el capítulo X de la tesis se busca el desarrollo de una formulación para la liberación acelerada de fármacos antirretrovirales por vía vaginal. La forma farmacéutica pensada para este fin son geles liofilizados. Esta formulación será denominada como discos vaginales, pues a los liofilizados se les dará una forma plana y circular, asemejándose a los *films*, aunque de mayor espesor. Estas dimensiones específicas permitirán mejorar su adhesividad y velocidad de disolución – debido a la mayor superficie expuesta a mucosa y medio, respectivamente – y facilitar su administración. Se decidió optar por liofilizados, pues la rápida rehidratación una vez administrada la formulación permitiría acelerar la liberación del principio activo.

Además, en este estudio se incorporó no solo el TFV, sino también la DPV. El hecho de diseñar una formulación que pueda albergar tanto un fármaco hidrófilo como uno lipófilo supone un reto en términos de desarrollo tecnológico. Además, la DPV ha sido tradicionalmente incluida en formulaciones de liberación sostenida – destacan los anillos vaginales cargados con este principio activo –, por lo que resultará de gran complejidad conseguir su rápida disolución en un medio acuoso, como es el fluido vaginal. Para resolver este problema se evalúa la influencia de la incorporación de agentes solubilizantes. Serán evaluados una ciclodextrina – 2-hidroxipropil-β-ciclodextrina – y dos tensioactivos, uno de naturaleza aniónica – lauril sulfato sódico – y otro de carácter neutro – polisorbato 60 –. También se estudiará la combinación de los tensioactivos con la ciclodextrina, pues han sido previamente referidas posibles interacciones entre estos componentes.

En una primera etapa del trabajo se lleva a cabo una caracterización de los geles preparados, de forma previa a su liofilización. Ya en los estudios de viscosidad y adhesividad de los geles es posible intuir una más que posible interacción entre la ciclodextrina y el lauril sulfato sódico cuando son combinados en la misma formulación, independientemente del fármaco incluido.

Los discos vaginales – geles liofilizados – también son caracterizados para conocer mejor su estructura y la modificación de la misma como consecuencia de la adición de los diferentes agentes solubilizantes. Los estudios de porosimetría revelan no solo la previamente expuesta interacción entre lauril sulfato sódico y 2-hidroxipropil-β-ciclodextrina, sino que además, como consecuencia de la adición de los fármacos, tienen lugar importantes modificaciones en la microestructura de los discos. Estas modificaciones han sido confirmadas mediante su observación visual, gracias a las imágenes tomadas por microscopía electrónica de barrido. Del mismo modo, se puede apreciar una incompatibilidad entre la ciclodextrina y el polisorbato 60 a las cantidades evaluadas. Los estudios para analizar las propiedades mecánicas de los discos reflejan que la incorporación de 2-hidroxipropil-β-ciclodextrina claramente mejora las características de resistencia y deformabilidad de los discos resultantes, pues le otorga al liofilizado una estructura más robusta y elástica.

En cuanto a los estudios de liberación del fármaco, quedó demostrado que la adición de lauril sulfato sódico a los discos vaginales, solo o en combinación con la ciclodextrina, permite la rápida liberación y disolución en fluido vaginal simulado tanto de TFV como de DPV. La combinación de 2-hidroxipropil-β-ciclodextrina y lauril sulfato sódico parece dar lugar a la mejor formulación posible, pues no sólo se consigue una excelente liberación acelerada de ambos fármacos, sino que mejora notablemente las características mecánicas de los discos. Además, la adición de ciclodextrinas a la formulación prolonga el tiempo de adhesión a la mucosa vaginal, mientras que la presencia de lauril sulfato sódico permite la formación de enlaces iónicos entre discos y mucina que otorgan una mayor fuerza de mucoadhesión que los enlaces iónicos formados cuando la interacción de la mucosa se produce con HPMC o 2-hidroxipropil-β-ciclodextrina.

Por lo tanto, estos discos vaginales han demostrado un gran potencial para el desarrollo de un microbicida vaginal de administración coito dependiente. Sin embargo, futuros estudios son necesarios para ajustar la dosis de los principios activos administrados a la mínima eficaz. En consecuencia, se podría reducir al máximo la cantidad de surfactante en la formulación, para garantizar que estos se encuentran en niveles seguros para su administración por vía vaginal.

Con este último capítulo se concluye el trabajo de investigación de esta tesis. A lo largo de los diez capítulos mostrados y discutidos anteriormente ha sido posible dar continuidad a una línea de investigación basada en el diseño, desarrollo y evaluación de formulaciones vaginales para la prevención de la transmisión sexual del VIH. Se han estudiado tres estrategias diferentes para lograr este objetivo – liberación prolongada, liberación pH-dependiente y liberación acelerada –. Aunque la mayor parte de la tesis ha sido desarrollada utilizando TFV como fármaco antirretroviral, para la última estrategia se ha querido abordar también la inclusión de DPV, dado el reto que supone la inclusión de un fármaco claramente lipófilo en formulaciones de liberación acelerada en un medio acuoso. El hecho de haber evaluado varias formas farmacéuticas y distintas estrategias de prevención se debe a que es necesario que las potenciales usuarias de estos microbicidas dispongan del mayor número de alternativas posibles, para así poder adaptar la posología para una mayor comodidad de la mujer. Del mismo modo, el conocimiento adquirido en cada uno de los capítulos de esta tesis ha podido ser interiorizado y aplicado a las siguientes etapas de desarrollo, contribuyendo no solo a una mejor actividad investigadora, sino a una formación más completa del investigador. Las formulaciones desarrolladas en la presente tesis, con el correspondiente apoyo para llevar la investigación a ensayos *in vivo*, podrían resultar en potenciales herramientas para prevenir la transmisión sexual del VIH.

Con vistas al futuro, la investigación en el desarrollo de microbicidas vaginales debe continuar con la misma intensidad – o incluso más – mostrada en la última década. Hasta el momento en que se logre una vacuna eficaz y accesible a gran parte de la población, la pandemia de VIH sólo puede ser controlada mediante la mejora del acceso a la terapia antirretroviral y a los tratamientos de prevención de la transmisión del virus. Como opinión personal, el futuro del desarrollo de microbicidas vaginales irá

innegablemente ligado a la inclusión de la nanotecnología en el desarrollo de estos productos, pues ya se encuentra cada vez más frecuentemente en las investigaciones desarrolladas en este campo. Además, el desarrollo de formulaciones inteligentes podrá dar lugar a microbicidas cada vez más avanzados y adaptados a las necesidades terapéuticas de las usuarias, lo que sin duda repercutirá en una mayor aceptación del sistema de prevención. Y es que, si algo se ha visto claramente en los ensayos clínicos desarrollados hasta la fecha, es que la adherencia al tratamiento profiláctico juega un papel fundamental en la eficacia del mismo.

CONCLUSIONES GENERALES

Los resultados de los estudios dirigidos al diseño, desarrollo y evaluación de microbicidas de administración vaginal para la prevención de la transmisión sexual del VIH permiten extraer las siguientes conclusiones:

1. Los microbicidas son una herramienta prometedora para la prevención de la transmisión sexual del VIH, aunque aún queda un largo camino por recorrer.
2. La gran cantidad de formulaciones evaluadas en las últimas décadas han dado lugar a más fracasos que éxitos, pero es crucial aprender de los errores que condujeron a formulaciones ineficaces para desarrollar un microbicida vaginal efectivo.
3. La adherencia al uso del microbicida ha sido uno de los principales obstáculos a la hora de conseguir una formulación eficaz en ensayos clínicos.
4. A corto plazo, los microbicidas basados en inhibidores de la transcriptasa inversa del virus – tales como tenofovir o dapivirina – son la mejor estrategia para el desarrollo de una formulación eficaz en ensayos clínicos.
5. Diversas formas farmacéuticas deben ser desarrolladas para asegurar que las mujeres disponen de múltiples opciones para protegerse en función de sus preferencias y necesidades.
6. Los comprimidos vaginales de liberación sostenida de tenofovir, capaces de conseguir una liberación prolongada del fármaco durante varios días, pueden disminuir la frecuencia de administración requerida y en consecuencia resultar en una mayor adherencia al tratamiento profiláctico.
7. Hidroxipropilmetil celulosa y quitosano son dos polímeros con potencial para lograr liberación sostenida de tenofovir, cuya combinación en comprimidos vaginales permite mejorar las propiedades de mucoadhesión y de liberación controlada.
8. Es posible elaborar granulados de tenofovir junto con Eudragit® RS, zeína o Gelucire® sin alterar la actividad del principio activo, y su incorporación a matrices hidrofílicas permite disminuir el hinchamiento de los comprimidos – haciéndolos más confortables para las usuarias –, así como prolongar el tiempo que permanecen adheridos a la mucosa.

9. La inclusión de gránulos preparados con tenofovir y Gelucire® 43/01 – en proporción 1:2 – en una matriz de hidroxipropilmetil celulosa y quitosano consigue prolongar el tiempo de liberación controlada del principio activo, logrando comprimidos con potencial para proteger a la mujer de la transmisión sexual del VIH mediante su administración semanal.
10. Los *films* vaginales son una forma farmacéutica que está atrayendo el interés de los investigadores debido a su gran versatilidad. Prueba de ello son las numerosas materias primas, técnicas de fabricación y tipos de *films* que es posible desarrollar. Es de esperar que en el futuro sea frecuente encontrar *films* vaginales inteligentes que permitirán adaptar el tratamiento a las necesidades terapéuticas, facilitando el día a día de la usuaria.
11. La combinación de HPMC y zeína en un *film* vaginal da lugar a una formulación que mejora las características de cada polímero; la liberación sostenida de tenofovir es posible gracias a la presencia de la zeína, mientras que la hidroxipropilmetil celulosa le otorga excelentes propiedades mecánicas.
12. La inclusión de plastificantes claramente altera el comportamiento de los *films*, modificando su flexibilidad y permeabilidad, así como la liberación de tenofovir.
13. Los *films* fabricados con hidroxipropilmetil celulosa y zeína – en proporción 1:5 – plastificados con un 40% de polietilenglicol 400 son una formulación bioadhesiva, con buenas propiedades mecánicas y capaz de liberar tenofovir de manera sostenida durante 5 días.
14. Los microbicidas sensibles a pH serían una herramienta de gran utilidad para proteger a las mujeres de la infección por VIH, acelerando la liberación del principio activo durante las relaciones sexuales.
15. La combinación de ácido oleico y polietilenglicol 400 en *films* de hidroxipropilmetil celulosa y zeína da lugar a una estructura única – debido a la orientación de la zeína en la presencia del ácido oleico – que es altamente beneficiosa para lograr una liberación pH-dependiente de tenofovir.

16. Los polímeros Eudragit® L 100 y Eudragit® S 100 serían excelentes excipientes para la fabricación de *films* vaginales de liberación pH-dependiente de tenofovir, ya que ambos son capaces de sostener la liberación del fármaco en presencia de fluido vaginal y disolverse en presencia del fluido seminal, logrando la completa liberación del principio activo.
17. Los discos vaginales basados en geles liofilizados de hidroxipropilmetil celulosa son una forma farmacéutica adecuada para la rápida liberación de fármacos por vía vaginal.
18. La combinación de lauril sulfato sódico y 2-hidroxipropil-β-ciclodextrina modifica la microestrucutra del gel de hidroxipropilmetil celulosa y produce sistemas liofilizados con poros más pequeños y menor volumen de poros, lo que no solo permite la rápida liberación de tenofovir y dapivirina, sino que también le otorga a los discos mejores propiedades mecánicas y mucoadhesivas.
19. La administración de discos vaginales – basados en geles liofilizados de hidroxipropilmetil celulosa y cargados con antirretrovirales – de forma inmediatamente previa a las relaciones sexuales podría ofrecer a las mujeres una herramienta confortable de protección frente al VIH. La cómoda autoadministración, y especialmente el uso a demanda, podría traducirse en una mejor adherencia al uso de este microbicida por las mujeres, que ha sido el talón de Aquiles observado en los microbicidas desarrollados hasta la fecha.

CONCLUSIONS

The results of the studies aimed at the design, development and evaluation of vaginal microbicides for the prevention of sexual transmission of HIV allow the following conclusions to be drawn:

1. Microbicides are a promising tool for the prevention of sexual transmission of HIV, although there is still a long way to go.
2. The large number of formulations evaluated in recent decades have led to more failures than successes, but it is crucial to learn from the mistakes that led to ineffective formulations to develop an effective vaginal microbicide.
3. Adherence to the use of the microbicide has been one of the main obstacles in achieving a formulation effective in clinical trials.
4. In the short term, microbicides based on virus reverse transcriptase inhibitors – such as tenofovir or dapivirine – are the most suitable strategy for developing an effective formulation.
5. Alternative pharmaceutical forms must be developed to ensure that women have multiple options to protect themselves based on their preferences and requirements.
6. Tenofovir sustained-release vaginal tablets, capable of achieving a prolonged release of the drug for several days, may decrease the frequency of administration required and consequently result in greater adherence to prophylactic treatment.
7. Hydroxypropylmethyl cellulose and chitosan are two polymers with the potential to achieve sustained release of tenofovir, whose combination in vaginal tablets improves the mucoadhesion and controlled release properties.
8. Tenofovir granules can be manufactured together with Eudragit® RS, zein or Gelucire® without modifying the activity of the active ingredient, and their incorporation to hydrophilic matrices allows to reduce the swelling of the tablets – making them more comfortable for users –, as well as to extend the time they remain attached to the mucosa.

9. The inclusion of granules prepared with tenofovir and Gelucire® 43/01 – in a ratio of 1:2 – into a matrix of hydroxypropylmethyl cellulose and chitosan delays tenofovir release, achieving tablets with the potential to protect women from sexual transmission of HIV through their weekly administration.
10. Vaginal films are a pharmaceutical form that is attracting the interest of researchers due to its great versatility. Proof of this are the numerous raw materials, manufacturing techniques and types of films that can be developed. In the future it may be frequent to find smart vaginal films that will allow to adapt the treatment to the therapeutic needs, making easier the day-to-day life of the patient.
11. The combination of HPMC and zein in a vaginal film results in a formulation that improves the characteristics of each polymer; sustained release of tenofovir is possible thanks to the presence of zein, while hydroxypropylmethyl cellulose gives it excellent mechanical properties.
12. The inclusion of plasticisers clearly alters the behavior of the films, modifying their flexibility and permeability, as well as the release of tenofovir.
13. Films fabricated with hydroxypropylmethyl cellulose and zein – in a 1:5 ratio – plasticised with 40% of polyethylene glycol 400 are a bioadhesive formulation, with good mechanical properties and capable of releasing tenofovir in a sustained manner for 5 days.
14. pH-sensitive microbicides would be a useful tool to protect women from HIV infection, accelerating the release of the active ingredient during sexual intercourse.
15. The combination of oleic acid and polyethylene glycol 400 in hydroxypropylmethyl cellulose and zein films results in a unique structure – due to the orientation of zein in the presence of oleic acid – which is highly useful in achieving a pH-dependent release of tenofovir.
16. Eudragit® L 100 and Eudragit® S 100 polymers would be excellent excipients for the manufacture of tenofovir pH-dependent vaginal release films, since both are capable of sustaining drug release in the presence of vaginal fluid and dissolving in the presence of the seminal fluid, achieving the complete release of the active principle.

17. Vaginal discs based on freeze-dried hydroxypropylmethyl cellulose gels are a suitable pharmaceutical form for the quick release of drugs vaginally.
18. The combination of sodium dodecyl sulphate and 2-hydroxypropyl-β-cyclodextrin modifies the microstructure of the hydroxypropylmethyl cellulose gel and produces freeze-dried systems with smaller pores and less pore volume, allowing not only the fast release of tenofovir and dapivirine, but it also gives the discs better mechanical and mucoadhesive properties.
19. The administration of vaginal discs – based on freeze-dried hydroxypropylmethyl cellulose gels and loaded with antiretrovirals – immediately before sexual intercourse could offer women a comfortable tool for protection against HIV. Comfortable self-administration, and especially use on demand, could translate into better adherence to the use of this microbicide by women, which has been the Achilles' heel observed in microbicides developed to date.